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Symposium
on
Enzyme Reaction
Mechanisms

PUBLISHED BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA
1959

SYMPOSIUM

ON

ENZYME REACTION MECHANISMS

GIVEN AT

RESEARCH CONFERENCE FOR BIOLOGY AND MEDICINE OF THE ATOMIC ENERGY COMMISSION

sponsored by

THE BIOLOGY DIVISION
OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee

held at

Gatlinburg, Tennessee
April 1 – 4, 1959

OAK RIDGE NATIONAL LABORATORY

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Introduction

Enzyme reaction mechanisms was the ıbject of the Twelfth Annual Research onference sponsored by the Biology Divi- on of Oak Ridge National Laboratory nd held at Gatlinburg, Tennessee, April -4, 1959.

The general purpose of this conference as to examine in detail basic enzymic ıechanisms concerned with the formation nd splitting of specific chemical bonds. he ORNL Biology Division, after many iscussions, concluded that an examina- on by those engaged in research in this nd closely related areas could lead to an wareness of new general principles relat- ıg chemical and enzymic reactions. This ubject proved to be a fortunate choice because several entirely new principles were brought out and formed the basis for a good part of the discussions during the meeting.

As in previous meetings, the conference was sponsored by the Biology Division of Oak Ridge National Laboratory with the cooperation of the Division of Biology and Medicine of the Atomic Energy Commission. Free and open discussion was encouraged, essentially all of which has been reproduced in this volume. A committee composed of Drs. S. F. Carson, W. E. Cohn, D. G. Doherty, G. D. Novelli, and Elliot Volkin prepared the program of this conference and gave valuable assistance to the editors during preparation of the manuscript.

Previous symposia in this series are:

1948—Radiation Genetics

1949—Radiation Microbiology and Biochemistry

1950—Biochemistry of Nucleic Acids

1951—Physiological Effects of Radiation at the Cellular Level

1952—Some Aspects of Microbial Metabolism

1953—Effects of Radiation and Other Deleterious Agents on Embryonic Development

1954—Genetic Recombination

1955—Structure of Enzymes and Proteins

1956—Biocolloids

1957—Antibodies: Their Production and Mechanism of Action

1958—Genetic Approaches to Somatic Cell Variation

ALEXANDER HOLLAENDER

ıtroduction to the Symposium on Enzyme eaction Mechanisms

ALEXANDER R. TODD
University Chemical Laboratory, Cambridge University, Cambridge, England

I do not propose to take up the time of e meeting by making a long opening dress, the more so since there are many ople here who have more important con- butions to make. Most of the points I sh to make will come up naturally in e course of discussions on individual pers. On looking through the abstracts the papers to be presented, however, I s struck by their variety and by the de- led information that is being presented. e mechanism of enzyme reactions is arly a subject of immense importance, t it is only in quite recent years that it s become profitable to meet and discuss as we are doing today. It has become ofitable because, on the one hand, the emists have progressed to a point where, be sure, they do not understand all out the mechanism of chemical reac- ns, but at least they can indicate what is ssible and what is impossible, and this a considerable help in considering alter- tive hypotheses. On the other hand, if ıay speak as an organic chemist, it has en gratifying to see how over the years biochemists have got around to pulling zymes apart to see how they work in- ad of relying, as in the old days, on what ght be called the "magic" approach to problem of enzyme reaction mechan- ı.

I suppose that I owe my presence here marily to the fact that, through my erests in the nucleotide field, I have be- ne deeply concerned with the chemistry organic phosphates in general—with at phosphates and polyphosphates do l how they do it—viewing it from a ctly chemical standpoint. When one ks at the subjects we are going to dis- s at this conference it is astonishing to l how almost all of them turn in one way or another on the behavior of derivatives of phosphoric acid. Phosphate and acyl transfer, carbon—carbon, carbon—nitrogen, and carbon—oxygen bond formation, peptide and protein synthesis—all these involve phosphate chemistry. And so it is perhaps appropriate that our first session should be devoted essentially to phosphate chemistry, for the basic part of nucleotide chemistry with which Dr. Khorana will be dealing is, in fact, phosphate chemistry.

In Cambridge my colleagues and I have been studying phosphate chemistry in one form or another for some fifteen years, and, as a result of our own work and that of others in the field, we have now reached a stage at which we can understand why certain compounds transfer phosphate, i.e., act as phosphorylating agents, how it happens, and we also know under what circumstances phosphates will act as alkylating rather than phosphorylating agents. We know how to phosphorylate using anhydrides and we know a number of phosphate derivatives that act as phosphorylating agents when protonated. Quite recently considerable interest has been aroused by the discovery of phosphates that transfer phosphate under oxidizing conditions and that may be significant in oxidative phosphorylation processes in biological systems. We have found, too, that there is a considerable difference between phosphorylation processes used in the laboratory to produce triesters of phosphoric acid (or fully esterified polyphosphates) and those designed to yield diesters of phosphoric acid (or partially esterified polyphosphates). I think most chemists have in the past too readily assumed (and to their disadvantage) that there was a common mechanism in all these phosphoryla-

tion processes. Although I shall not discuss the matter in detail here, I would suggest that processes designed to yield diesters of phosphoric acid, i.e., those in which the phosphorylating agent is derived from a monoester of phosphoric acid, involve as the active reagent monomeric metaphosphate.

When we turn from laboratory experiments to consider the biochemical or biological behavior of phosphates, it is, I think, fair to say that one can now see in a rough way just what is going on in a great many enzyme reactions involving phosphates. There are, however, some difficulties in explaining certain features. One difficulty that has always interested me is that in biological systems nature uses mono- or diesters of polyphosphoric acids (e.g., adenosine triphosphate) to carry out phosphate transfer or exchange reactions, whereas we know that in the laboratory such transfer reactions are only possible with fully esterified polyphosphates. Such mono- and diesters of polyphosphoric acids are not normally labile or reactive in the general *p*H and temperature ranges encountered in living organisms. All the phosphate transfer and exchange reactions involve attack by a nucleophile on phosphorus, and it is clear that in partially esterified phosphates or polyphosphates approach of the nucleophile will be hindered or wholly prevented by the negative charge associated with the partly esterified compounds. Only when these negative charges are removed by, for example, esterification does nucleophilic attack become feasible. One of the secrets of the enzymes is that they must have a means of affecting partly esterified phosphates and polyphosphates in such a way as to depress ionization (I speak now quite generally) at appropriate points in the molecule so that it behaves in a manner akin to that of a fully esterified compound. Exactly how they do it is a matter for discussion.

There are experimental observations—we in Cambridge have made a number of them—on the use of the so-called inclusion compounds with cyclodextrins as models for enzyme systems that show that such compounds as adenosine pyrophosphate and diphenyl pyrophosphate can in this way be made labile. It is possible that one very important function of the large p tein molecules in enzyme systems is stric analogous to that of the cyclodextrin these model experiments. The possibil seems to be at least worthy of further ploration, particularly since, if it pro valid, it would offer a partial explanati for specificity in action. For it would possible by using different proteins to press in varying degree the ionization o phosphate and so cause it to react pref entially with one nucleophile. It would a help to explain why it is necessary to u large protein molecules in enzyme syster for clearly it is only with really large mo cules that such effects would be eas realized. If sheer size is an importa feature of enzymes the outlook is perha a little depressing for the organic chem for the bigger the molecule the more d ficult a substance is to study chemically

I look forward to hearing more about various views on these important top during our meeting and I would like a to consider the relation between views the type I have mentioned and the questi of active sites on enzymes. I sometim wonder whether active sites are universa important or whether they are alwa clearly definable. There are probably w differences between enzymes in this spect and I am not sure whether in so cases precise definition of active sites w lead to advances in our knowledge co mensurate with the effort involved in fining them. But I am open to argument this point!

It is now almost time for me to ha over to our first speaker but before doi so there is one further thing I would l to say. Later this morning Dr. Mildr Cohn is going to tell us something abo her physicochemical studies on phospha It gives me very great pleasure to see here and to learn of her recent work, there is much to be learned from quantitative study of phosphate reacti by kinetic methods. I feel most stron that there has been too little of this ty of work in the past. Recently there been increased interest shown by the ph ical chemists in the field, but there room for much more. Without quantitat studies of this type real progress will difficult. But, if the physical chemist w

ovide the data with which to weld to-
ther the more qualitative observations of
e organic chemist and the biochemist,
en we can look forward to a rapid clarifi-
tion of many vexing problems in the
zyme field.

And now to our program! We begin
th a paper by Dr. Khorana on the syn-
esis of polynucleotides. It is a particu-
lar pleasure for me to welcome him here as one of my former students. I have watched the beautiful work he has been doing in Vancouver with the greatest interest and I am, like all of you, looking forward to hearing something of his current work and thoughts on polynucleotide chemistry.

ynthesis and Structural Analysis of Polynucleotides

H. GOBIND KHORANA
British Columbia Research Council, Vancouver, Canada

It will probably be generally agreed that major achievement of modern biology the recognition of the vital functions of ıe nucleic acids and of the close interre-tions of nucleic acids and proteins. Com-ete understanding of these intricate re-tionships and adequate experimental ısis for any "information" theory will be ovided only by complete structural corre-tions of the two classes of macromole-ıles. Therefore, the problems of the etailed structural analysis (end-group and quential analyses) of the nucleic acids ssume importance at once and, although ıe progress in the corresponding problems the polypeptide field has been impres-ve, little has been accomplished so far ith the nucleic acids. A few years ago ır Laboratory undertook a rather ex-nded program of research in this field, ith the first major aim of applying the chniques of organic chemistry to the nthesis of polynucleotides. We envis-ged the use of synthetic polynucleotides completely defined structures for a host chemical, physicochemical, and enzymic udies with the hope that these basic udies would pave the way for tackling the gantic problems of sequential analysis the nucleic acids.

SYNTHESIS OF POLYNUCLEOTIDES

Synthesis of internucleotide bonds. The st major requirement is a satisfactory ethod for formation of the phospho-ester bonds between different nucleo-des. Two approaches were previously sed for synthesizing diesters of phosphor- acid. The first one was used by Baer 55) and coworkers in their work on ıospholipids. This utilizes a bifunctional ıosphorylating agent such as monophenyl ıosphorodichloridate in the stepwise ıosphorylation of two different hydroxy- compounds. The second approach, which was used in the first synthesis of dithymidine dinucleotide (Michelson and Todd, '55), involves the preparation of a nucleoside phosphite, its conversion to a phosphorochloridate, and the subsequent phosphorylation of a second suitably protected nucleoside.

Neither of these approaches is satisfactory for practical syntheses of polynucleotides. The method that we have developed and used in all our work consists in reacting a monoalkyl phosphate (I) with an alcohol under anhydrous conditions with dicyclohexylcarbodiimide (DCC) or *p*-toluenesulfonyl chloride according to figure 1.

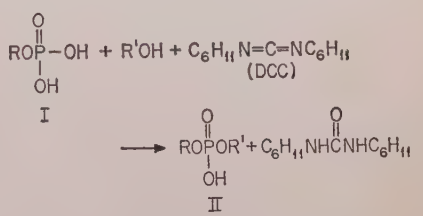

Figure 1

The mixed diester of phosphoric acid (II) is thus obtained directly and the yields are consistently high. The mechanism of this reaction is rather complex and has been discussed briefly elsewhere (Gilham and Khorana, '58; Smith *et al.*, '58). In the nucleotide field, the reaction was first applied to the synthesis of thymidylyl-(3′ → 5′)-thymidine (VI; fig. 2). The desired starting materials, 5′-*O*-tritylthymidine (III) and 3′-*O*-acetylthymidine 5′-phosphate (IV), were prepared in excellent yield by the tritylation of thymidine and acetylation of thymidine 5′-phosphate, respectively. The dinucleoside phosphate (VI) was obtained in about 65% yield when equimolar amounts of the two nucleoside components were used. Later experiments carried out with J. P. Vizsolyi showed that its yield is quantitative with

respect to III if two molar equivalents of the nucleotide IV are used and, again, it is quantitative with respect to the latter if a corresponding excess of the former is used. This reaction was then applied to syntheses of the mixed dinucleoside phosphates, thymidylyl-(3′ → 5′)-deoxycytidine and thymidylyl-(3′ → 5′)-deoxyadenosine, as shown and both were obtained in satisfactory yields (Gilham and Khorana, '58, '59).

Stepwise synthesis of mixed deoxyribooligonucleotides. Stepwise synthesis of oligonucleotides containing different mononucleotide units requires selective unblocking of a hydroxyl function at one end of the fully protected dinucleoside phosphates, to give substances like VII and VIII and condensation of these with another suitably protected nucleotide. So far two trinucleotides have been prepared in this way (fig. 3). Thus mild alkaline treatment of 5′-*O*-tritylthymidylyl-(3′ → 5′)-(3′-*O*-acetyl)-thymidine (V) gave VI which was reacted with two molar equivalents of IX. After the protecting groups were removed, trithymidine diphosphate (XI) was obtained in about 68% yield. Similarly, mild alkaline treatment of 5′-*O*-tritylthymidylyl-(3′ → 5′)-(3′-*O*-acetyl)-*N*-acetyladenosine gave VIII, which was reacted with X to give the mixed trinucleotide XII containing the three bases thymine, adenine, and cytosine in the order (Gilham and Khorana, '59). Synthesis of higher and various mixed oligonucleotides is certainly feasible and is in progress. However, the present work has brought into sharp focus certain problems that must be solved if efficient syntheses of higher oligonucleotides are to be realized; these are (1) insolubility in anhydrous organic solvents, (2) separation of products, (3) extreme acid lability of glycosyl bonds.

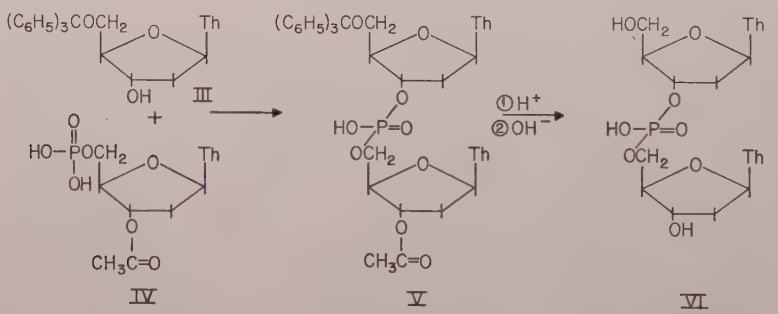

Figure 2

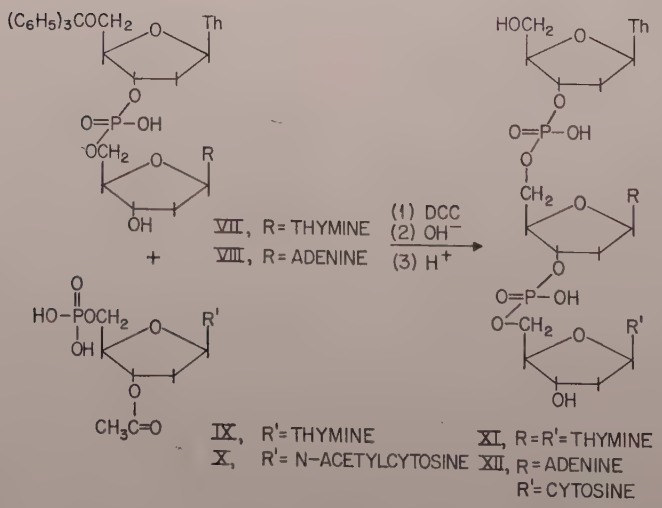

Figure 3

the purine deoxyribonucleosides, and
) the reactivity of the amino groups on
e adenine and cytosine ring.

Introduction of phosphomonoester oups at termini. Linear polynucleotides ually carry a phosphomonoester group one or the other end of the chain and e methods mentioned can be adapted to eld such "true" oligonucleotides. Two ap- oaches are available. The first one is at in which a protected phosphoryl oup is used as a blocking group on a ıcleoside at the outset of the synthesis. number of dinucleotides bearing 5′-phos- ıomonoester end groups have been syn- esized by this method (Gilham and ıorana, '58).

In the second approach, the phospho- onoester end group is introduced after e formation of the internucleotide bonds. ıus, for example, VII may be phosphoryl- ed by reaction with β-cyanoethyl phos- ıate and DCC to give the 3′-cyanoethyl phosphate derivative of VII, from which the cyanoethyl group is removed by alkaline treatment and the trityl group by acidic treatment to give thymidylyl-(3′ → 5′)-thymidine 3′-phosphate. The new method of phosphorylation was developed by Dr. Tener in our Laboratory (Tener and Gilham, '59).

Specific synthesis of C_3'–C_5' interribonucleotidic linkages. The specific synthesis of C_3'–C_5' interribonucleotide linkage is seriously complicated by the *cis*-(2′)-hydroxyl group in the ribonucleosides. The requirement of any general approach to the problem is to block selectively the 2′-hydroxyl function with a group that will not migrate to the 3′-hydroxyl function and that may be removed at the end without damage to the phosphodiester linkage synthesized. Such an approach is outlined in figure 4 and was recently used successfully in the first synthesis of a C_3'–C_5' linked diribonucleoside phosphate (XIX)

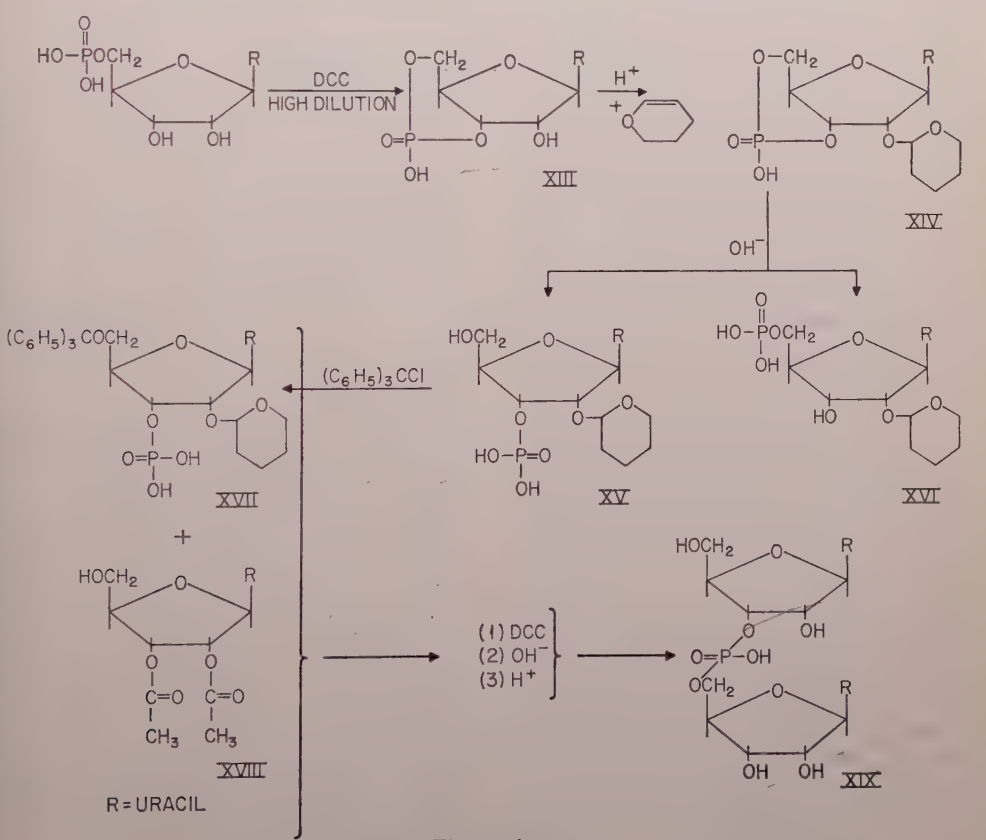

Figure 4

(Smith and Khorana, '59). Uridine 5′-phosphate is converted in high yields to XIII (R = uracil) on reaction in extremely dilute solution with DCC. Reaction of this cyclic phosphate, as the free acid, with dihydropyran in dioxane gives XIV quantitatively, which hydrolyzes on being heated in alkali to a roughly 4:1 mixture of XV and XVI. Although the mixture can be separated on an ion-exchange column, a better approach is to treat the mixture with triphenylmethyl chloride in pyridine. Only XV reacts, and the product (XVII) can be readily separated from XVI by partition chromatography. The condensation of XVII with XVIII under standard conditions followed by mild alkaline and acidic treatments to remove the protecting groups gives XIX, which has been thoroughly characterized chemically and enzymically.

Polymerization of mononucleotides. Stepwise synthesis of mixed oligonucleotides will undoubtedly be essential ultimately but is beset with great difficulties. Polymerization of mononucleotides is an alternative, more expedient, means of obtaining a range of simple polynucleotides that serve admirably for many studies.

In this method of diester synthesis, it was to be expected that treatment of an unprotected nucleotide with DCC under the standard conditions would give polymers. Thymidine 5′-phosphate was used in initial studies, and polymeric products were indeed obtained. The first problem was one of separation and isolation of pure components, and this was solved, as described in detail elsewhere (Tener *et al.*, '58), by chromatography on ECTEOLA cellulose columns followed by preparative paper chromatography. Since the means of identification of the major products have been described in detail (Tener *et al.*, '58), they will be mentioned only briefly. Two series of homologous oligonucleotides were obtained. The first are the linear oligonucleotides, which contain the repeating naturally occurring phosphodiester linkage, a 5′-phosphomonoester group at one end and a 3′-hydroxyl group at the other. The second series of compounds are the cyclic oligonucleotides that arise by end-to-end intramolecular cyclization of the linear oligonucleotides. The extent of the cyclization process that compe with linear polymerization decreases w increase in chain length. Thus, where at the dinucleotide level the cyclic meml is much more abundant than the lin dinucleotide, at the pentanucleotide st the cyclic compound forms only a sm portion of the total fraction. The 3 cyclic phosphate of mononucleotide, wh can be regarded as the monomeric me ber of the cyclic oligonucleotides, was a present in a small amount in the synthe mixtures (Tener *et al.*, '58).

The cyclization reaction, although teresting, is wasteful for linear polymeri tion. A procedure that greatly redu this reaction has been devised. This c sists in adding 25–50% by concentrati 3′-O-acetylthymidine 5′-phosphate to t midine 5′-phosphate before polymeri tion. The protected nucleotide forms t terminating unit of the greater portion polymeric mixture and the linear polym are therefore unable to undergo end-to-e cyclization. The acetyl group is sub quently removed with alkali. The sa principle (copolymerization of one p tected nucleotide with a different "fr nucleotide) was used to obtain a use series of compounds, homopolymers t minated in a different nucleotide at one the ends. Thus, by copolymerizing N_4 diacetyldeoxycytidine 5′-phosphate a thymidine 5′-phosphate, linear thymidi oligonucleotides terminating in deoxyc dine were obtained. The advantages such oligonucleotides in many structu studies are obvious.

In further extension of the polymeri tion work, thymidine 3′-phosphate yield another series of linear thymidine oli nucleotides whose characteristic is t they bear 3′-phosphomonoester end grou (Turner and Khorana, '59). Studies a also in progress on the polymerization purine deoxyribonucleotides. Indeed, t work can be extended in a number directions, and a host of questions rega ing polymerization remain to be answer For example, how far can chemical po merization be induced to go? What the kinetics and precise mechanism polymerization reaction? Practically, would be particularly interesting to stu the polymerization of preformed di- a

nucleotides. For example, polymerization of adenylic-thymidylic dinucleotide would give polymers containing these two mononucleotides in alternating sequence. Undoubtedly, a large number of basic physicochemical studies in the polynucleotide field are possible, opportunity for which is provided by the synthetic polymers of small size. Studies of this type now appear imminent.

STRUCTURAL ANALYSIS OF POLYNUCLEOTIDES

Using synthetic oligonucleotides, we are investigating possible approaches to the end group and sequential analyses of polynucleotides. It seems clear at the outset that maximum progress will accrue from a combination of chemical and enzymic approaches as has been amply demonstrated in the protein field (Khorana, '52; Sanger, '52). Both these major lines are being concurrently investigated. Chemically, efforts are being devoted initially to the marking of end groups in polynucleotide chains. The distinguishing features of the end nucleotides will be either a free hydroxyl group or a phosphomonoester group. Possible methods for marking such end groups are proposed. In the ribopolynucleotides, a 2′,3′-*cis* pair of hydroxyl groups may be present. A method for stepwise degradation based on the selective periodate oxidation of such a pair of hydroxyl groups was proposed previously (Whitfeld, '54; Brown *et al.*, '55).

Chemical marking of end groups

Acetylation of 3′-hydroxyl groups. The technique that has been investigated with thymidine oligonucleotides as models consists in acetylation of the end 3′-hydroxyl function by acetic anhydride–pyridine mixture and subsequent degradation by crude snake venom. The diesterase first cleaves all the diester bonds and the mononucleotides released are dephosphorylated to the corresponding nucleosides by the 5-mononucleotidase also present in the venom. The end unit present as the acetylated nucleotide is, however, not attacked by the mononucleotidase since this enzyme absolutely requires the 3′-hydroxyl group to be free. It is thus possible, in principle, to determine the end group in a polynucleotide chain. The main problem here is the development of a satisfactory technique for selective acetylation of the end hydroxyl group of mixed polynucleotides.

Methylation of phosphomonoester end groups. A procedure for selective methylation of phosphomonoester end groups (Khorana, '59) based on work reported by Smith *et al.* ('58) has been devised. A dilute solution of trialkylammonium salt of an oligonucleotide is treated in methyl alcohol with DCC at room temperature. The diester linkages are inert but the phosphomonoester groups react to form the monomethyl esters.

In the ribooligonucleotides, no cleavage of the internucleotide linkage was observed. In principle, the only class of polynucleotides where the technique will not apply directly is represented by the partial structure XX (fig. 5). Here reaction will give preferentially the terminal cyclic phosphate structure XXI. However, as was shown by Tener and Khorana ('55) for the simple ribonucleoside 2′,3′ cyclic phosphates, it should be possible to convert XXI to an acyclic methyl ester XXII by treatment with methyl alcohol and mild acid.

By the use of enzymes to be described, we found it feasible to identify the terminal nucleotide units bearing methylated

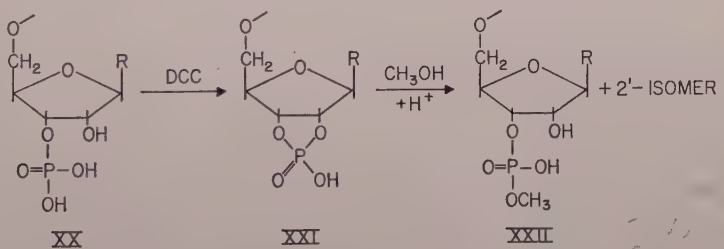

Figure 5

phosphate groups of oligonucleotides. Large polynucleotide chains could be degraded after methylation by use of appropriate nucleases and phosphodiesterases in combination. Solubility of the nucleic acids is not expected to be a serious problem in methylation. The formation of tri-*n*-butylammonium salts has a solubilizing effect, and a number of nonaqueous solvents, including alcohols, have been reported for DNA and RNA. A further advantage of methylation will lie in the use of methyl alcohol labeled with C^{14} or H^3.

The mode of action of phosphodiesterases and nucleases

The enzymes attacking nucleic acids (phosphodiesterases) may be classified into two major groups. The first group comprises specific phosphodiesterases such as RNases and DNases. Enzymes that attack both ribo- and deoxyribopolynucleotides are simply called phosphodiesterases. The main problems here are (1) to obtain reliably pure preparations of enzymes and (2) to determine their mode of action and specificity as precisely as possible so that they may subsequently be used in sequential work on unknown polynucleotides. The work described here concerns two phosphodiesterases—namely, those venom and spleen. Ribonucleases will mentioned only briefly and some rece work on pancreatic DNase will be includ

Venom and spleen phosphodiesteras These two enzymes have previously be shown to attack internucleotide bonds both ribo- and deoxyribopolynucleotid the products from the venom diestera being nucleoside 5′-phosphates where those obtained from spleen are nucleosi 3′-phosphates. A number of procedu have been described for purification of t venom diesterase. The preparation us in most of our work (Razzell and Khora '58, '59a, b) was obtained by the aceto fractionation procedure of Koerner a Sinsheimer, followed by chromatograp on a DEAE cellulose column accordi to Boman and Kaletta and represent about 70-fold purification.

The spleen diesterase was studied Heppel and Hilmoe ('55), and a purifi tion procedure was made available to by Dr. Hilmoe. The series of compoun first studied with the two enzyme prepa tions were the linear thymidine oligo cleotides from which the 5′-phosphomo ester end groups had been removed treatment with phosphomonoestera

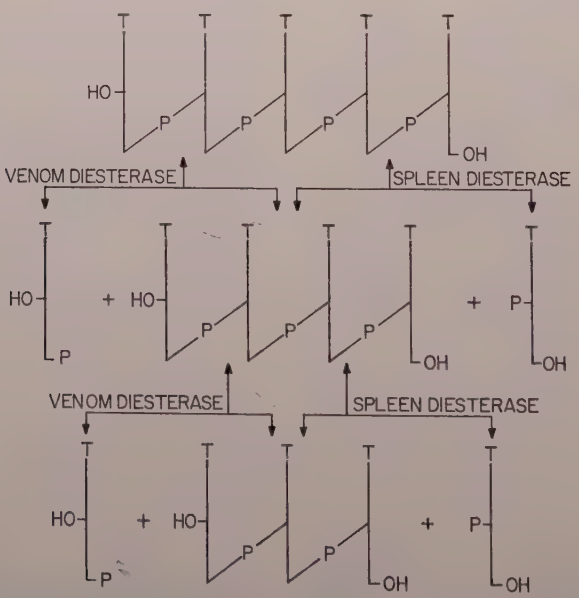

Fig. 6 Schematic representation of the degradation of thymidine oligonucleotides by snake venom and spleen phosphodiesterases.

netic study with paper chromatography owed that the action of the venom diesase on such compounds is stepwise and gins from the end of the chain bearing 3′-hydroxyl group; attack on the ternal phosphodiester bonds liberates succssively thymidine 5′-phosphate and the ver homolog. The process continues and ymidine appears only toward the end of gradation. Corresponding study of the ion of spleen phosphodiesterase again owed that the degradation was stepwise t complementary to that of the venom sterase in that it proceeded from the d of the chain bearing a 5′-hydroxyl oup and resulted in the successive liberan of thymidine 3′-phosphate residues d the lower homologs. These findings e illustrated schematically in figure 6, ere shorthand formulations are used the oligonucleotides.

Out of the extended studies carried out h venom diesterase (Razzell and Khoa, '59a, b) only two points will be ntioned here. (1) Di- and higher oligucleotides bearing 5′-phosphate end oups are hydrolyzed much faster than the corresponding members lacking 5′-phosphate end groups. Yet the hylysis of the oligonucleotides bearing 5′osphomonoester end groups has been nclusively shown to begin from the far d, i.e., the end bearing the 3′-hydroxyl oup. A simple interpretation of this acating effect of the 5′-phosphomonoer end group is that it merely serves to ver the 5′-hydroxyl group and so prents the enzyme from binding at the rong" end. (2) The "exopolynucleotise" type of action of the enzyme is not solute since cyclic oligonucleotides, ich lack terminal groups, are also slowly drolyzed. In addition, oligonucleotides aring 3′-phosphomonoester groups, ich are hydrolyzed even more slowly, are acked rather randomly at points within polynucleotide chain. Although the ssibility must remain that these weak tivities are caused by a second contamiting enzyme, it does appear from our dies that the same enzyme is responsi for all the activties. Nevertheless, en the right type of end group (3′-hyoxyl group in deoxyribopolynucleotides d 2′- and 3′-hydroxyl groups in ribopolynucleotides), the enzyme offers promise for sequential analysis of polynucleotides. The endopolynucleotidase action is insignificant for oligonucleotides of this type and it will probably be reduced further owing to the hydrogen-bonded internal structure of larger polynucleotides. Results appearing from a number of laboratories have confirmed these views.

Ribonucleases. Since the clarification of the mode of action of pancreatic ribonuclease, a number of related ribonucleases have been studied (see Shuster *et al.*, '59, for references). Markham and Smith ('52) isolated and identified a large number of di- and trinucleotides in their original studies of the action of pancreatic RNase on certain ribonucleic acids. Reddi ('59) has reported similar studies on tobacco mosaic virus RNA. Such studies may be of value in obtaining indications of the gross structural patterns of the ribonucleic acids of different origin.

Deoxyribonucleases. Pancreatic DNase is the oldest known member of this group of enzymes, several of which have been described in recent years. Its major characteristic is that its action on DNA produces fragments all of which contain 5′-phosphomonoester end groups. On the other hand, DNase of spleen and thymus origin, for example, form fragments bearing 3′-phosphomonoester end groups. Beyond this the mode of action of this group of enzymes has remained unknown. Some of our recent studies on pancreatic DNase with synthetic substrates bear on the subject. Previous studies on DNA showed that the products consisted of 1% mononucleotides, 15–18% dinucleotides, and the remainder of the material was a complex mixture of higher oligonucleotides. Potter *et al.* ('58), using a large amount of the enzyme, found that the trinucleotide deoxyadenylyl - (3′ → 5′) - deoxyadenylyl-(3′ → 5′)-thymidylic-(3′) acid was hydrolyzed to thymidine 3′,5′-diphosphate and deoxyadenylyl-(3′ → 5′)-deoxyadenosine. The compounds studied by us are listed together (XXIII–XXIX) by shorthand formulations, even though some of them have already been mentioned (fig. 7).

Compounds XXIII–XXV were not degraded under the standard conditions of digestion with the enzyme. Interestingly

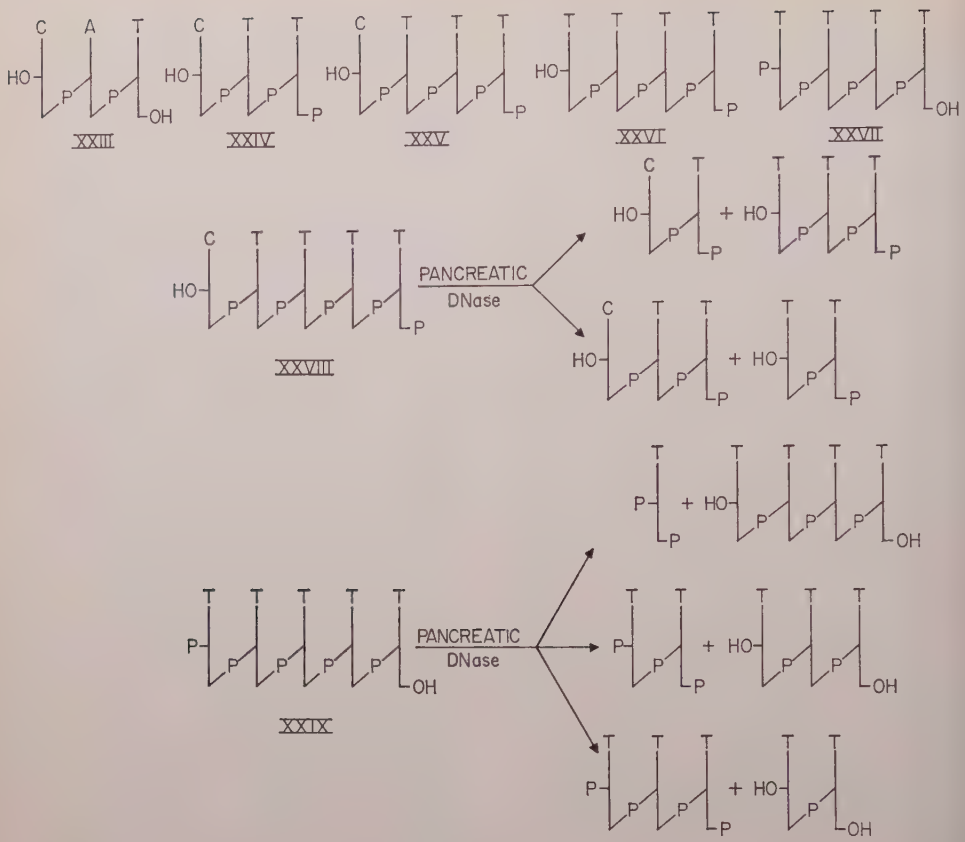

Figure 7

enough, the higher homolog of XXV (i.e., XXVIII) was attacked at each of the two innermost bonds to give the products shown.

Although the thymidine tetranucleotide bearing a 5′-phosphate end group (XXVI) was resistant, the isomer bearing a 3′-phosphate end group (XXVII) was degraded, apparently, mainly at the central bond, although these results have to be confirmed. The pentanucleotide XXIX was attacked again at multiple points.

Results to date thus show (1) the effect of the 3′-phosphomonoester end group, (2) the minimum size of the oligonucleotide in a homologous series necessary for the enzyme action to occur, and (3) that the action, where it does occur, is often at multiple points.

In conclusion, the point that I should like most to emphasize is that the progress-determining factor in the structural and sequential analyses is going to be ou ability to separate mixtures of polynu cleotides. It is encouraging to note in th connection that the rather low molecula weight "soluble" RNA, which accepts a tivated amino acids, has become the focu of interest, and that several groups workers are now engaged in devising pr cedures for its fractionation.

ACKNOWLEDGMENT

In the work that I have described, has been my good fortune to be associate with a number of very able and dilige colleagues: Doctors Gordon M. Tene Peter T. Gilham, W. E. Razzell, Micha Smith, Alan F. Turner, and Burkha Lerch, and Mr. John P. Vizsolyi. I a greatly indebted to all of them for the sustained efforts in an unusually difficu field of experimental organic chemistr

OPEN DISCUSSION

TODD[1]: There are two points I would ike to raise now.

First, the last part of your talk on the ction of enzymes on the synthetic poly- nucleotides I find very interesting, but n the case of the DNase studies the re- ults, in addition to being interesting, are lso rather confusing and slightly disturb- ng. For example, in the tetrathymidine derivatives you mentioned the enzyme vould not work when the phosphate was n the 5 position in the terminal group but vould work when it was in the 3 position. f DNA is synthesized by a mechanism like hat of the Kornberg enzyme, we would ex- pect the finished molecule to have a termi- nal 5-phosphate group and not a 3-phos- phate group. In this respect your findings do seem peculiar and, although I realize hat the answer may come from further work, I wonder if you have any comments o make on it.

The second point is one you may or may not have considered. I think what we want is an enzyme that will not just break off little bits from the end of a polynucleo- tide chain or simply break it into perhaps two pieces; we want one that will break the chain into large chunks in a definite way, because, even with the use of tracers, we will be straining chemical degradation methods to the uttermost limits if we have to start end-group or sequence determina- tions on enormously long chains such as are found, we are told, in the natural nu- cleic acids. Of course, it may be that the molecular weights of the natural nucleic acids have been overestimated, but if the estimates are anywhere near the truth, then it is going to be very difficult to do satisfactory sequence determinations with- out first breaking up the molecule in a definite manner.

KHORANA: A number of imporant ques- tions have been raised by Professor Todd. Let us take them briefly one by one. First there is the specificity of DNase. Here I think we may really be delineating the minimum size required for the enzyme ac- tion. The work as it stands is really not directly relevant to the use of the enzyme for DNA degradation but is an attempt to define the mode of action of the enzyme. From the extensive previous work on the degradation of DNA by this enzyme we know that DNA is actually the best sub- strate and also that it is possible to halt the action at early stages of degradation.

But in regard to the larger chunks, I must emphasize that the progress-deter- mining factor here is really going to be the methods for separation of these larger chunks. Until we know how to separate these it is really going to be impossible to get the whole sequence, especially since there are only about four nucleotides. However, a start on these difficult prob- lems must be made and if one end can be tagged, say the phosphomonoester group, of a polynucleotide chain, then it should be possible to degrade the chain by the use of appropriate enzymes and rigorously purify the small fragments containing the label. In this way we should get the se- quences at and near a terminus of a chain. I think that this is the best we can do to begin with. Also, I am optimistic enough to think that perhaps with vigorous effort, particularly on smaller polynucleotides, we may discover some new principles of separation of polynucleotides. Finally, I must emphasize the place of enzymes act- ing stepwise from ends among the means available for work on sequential analysis. Having isolated a small chunk, the best we could do would be to use such enzymes.

ATWOOD[2]: From what Dr. Khorana has said, it is obvious that the possibility of getting sequences in nucleic acids is imminent. This raises the question how to get a starting material that we have some reason to believe is homogeneous with respect to its sequence. I do not know of any biological source, and I do not think that any of the means of fractionation of natural nucleic acids can—even in princi- ple—lead to the isolation of a nucleic acid of homogeneous sequence. I would be very glad to hear of any ideas about this; it is obvious that the problem will require a lot of thought.

TODD: Yes, I was about to hand this one straight over to Dr. Khorana, although I suspect that he is a little like me. I would be very glad to hear the answer to that one, too.

[1] Alexander Todd, University Chemical Laboratory, Cambridge, England.

[2] K. C. Atwood, University of Chicago.

KHORANA: Yes, I am sure I would also; but there is a ray of hope. I think that perhaps we should first work with the low-molecular-weight soluble RNA, for which estimates of molecular weights of about 10,000 have recently been made and which therefore contains no more than say 30 nucleotide units. I hope that the workers now engaged in its separation will show perseverance.

There is another ray of hope. I think that in certain small phages the calculations indicate that there may not be more than a few species of nucleic acids. Then there is, in particular, the ϕX174 virus with which R. L. Sinsheimer has been working that is assumed to be a single-stranded DNA of molecular weight 1.8 millions. If this is true, at least we may have the starting material for this work.

HAGER[3]: One possibility that comes to mind with regard to the sequence problem is that, with the availability of synthetic dinucleotides, it should be possible, at least in theory, to use the classical enrichment culture technique to prepare a whole battery of enzymes that could serve as analytical tools. Ideally, it should be possible to obtain enzymes that would hydrolyze only the 3,5 phosphodiester linkage between specific pairs of bases.

KHORANA: That is an interesting idea. We would of course have to wait until we have a larger range of oligonucleotides and also much greater quantities.

CALVIN[4]: I should like to suggest another method of separating at least small to medium size polynucleotides—a method I have not yet seen described or even suggested. You all are familiar with the extreme power of solvent-solvent extraction as developed by L. C. Craig in his multitube machines. Unfortunately, most of the polynucleotides have only one solvent, namely, water. It would be desirable if we could devise a system in which there would be two water phases that did not mix. This is possible. With suitable additions of polymers two immiscible water phases can be made, both of which are more than 90% water. It is conceivable that such two-phased liquid systems could be developed that would allow us to put nucleotide and polynucleotide mixtures through a Craig machine and thus get the advantage of this enormous sepa ation ability.

BOMAN[5]: This is in progress and ha already been partly done. Dr. P. A. A bertsson in Uppsala has worked out sever two-phase systems and has tried them o viruses, cell particles, and proteins, an we have also started to try it on the d gestion products from DNA.

SCHMIDT[6]: What are the systems?

BOMAN: It is a two-water phase syste with different polymers.

SCHMIDT: I mean, specifically what ar the polymers?

BOMAN: One polymer is methylcellu lose and the other is dextran (Albertsso '58).

TODD: Quite a long time ago we trie various solvent mixtures for this purpo but we did not have a great deal of su cess. We have not done much since, bu our observations on the formation of i clusion compounds in aqueous solutio of cyclodextrins seem to form a possib basis for developing systems similar those you have been discussing. I wond whether you have found systems of th type effective in Sweden?

BOMAN: Albertsson and I have trie this on digestion products from DN This work was, however, not complete before I left but it seems to be a possib way.

SCHREINER[7]: We have tried this syste in trying to separate enzyme proteins an have found one drawback to be that th partition coefficients of many proteins i the methylcellulose-dextran system ar close to 1; it is a question whether we ca fruitfully use this approach, but it ma be that in the case of nucleic acids th partition coefficients are sufficiently di ferent from 1 so that an efficient system fractionation may be obtained.

CALVIN: The enormous advantage th I have in mind is the variety that can introduced into the two phases. By a justing salt concentration, polymers, m

[3] L. P. Hager, Harvard University.
[4] Melvin Calvin, University of California.
[5] H. G. Boman, The Rockefeller Institute f Medical Research.
[6] Gerhard Schmidt, Tufts College Medic School.
[7] H. R. Schreiner, The Linde Company, Tor wanda Laboratories.

cular weight, and that sort of thing, you an vary the distribution at will.

METZENBERG[8]: I would like to mention chance observation. In the case of RNA, very insoluble salt is formed in water ith cetyltrimethylammonium bromide, hich is a cationic detergent. Surprisingly ough, this rather poorly dissociated com- lex is soluble in alcohol and it is even oluble in alcohol containing ether. It eems possible that, because this complex soluble in organic solvents, there would e some possibility of fractionating RNA y countercurrent partition.

[8] R. L. Metzenberg, University of Wisconsin.

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Mechanisms of Enzymic Cleavage of Some Organic Phosphates[1]

MILDRED COHN[2]
Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri

The variety of phosphate esters and related compounds that appear as structural units and as metabolic intermediates has stimulated interest in the mechanisms of their transformations. To the biochemist, phosphate transfer is important because it is the chief pathway of energy transfer from one metabolic process to another.

Ideally, we would like to have sufficient information to describe these enzymic reactions in detailed mechanistic terms involving intermolecular forces. Attainment of that goal would necessitate not only a knowledge of the true substrate and the nature of the transient intermediates but of the active site of the enzyme as well. In spite of the rudimentary state of our knowledge of these factors for most enzymic phosphate reactions, considerable progress has been made in eliminating many of the theoretically possible mechanisms.

Only in recent years have a number of laboratories initiated systematic investigations of the mechanisms of nonenzymic reactions of organic phosphates, particularly hydrolysis and other solvolyses. The multiplicity of mechanisms observed and postulated for hydrolysis alone is frightening (Barnard *et al.*, '55). For example, in the hydrolysis of monosubstituted phosphate esters, first- and second-order attacks may occur, hydrolysis may occur by a displacement reaction on carbon or phosphorus (i.e., with C—O or P—O cleavage) and with each of the ionic species of the substrate. Furthermore, hydrolysis may theoretically occur by addition to the P=O bond to form a quinquevalent phosphorus intermediate or by cleavage to form an unstable metaphosphate intermediate.

On the other hand, the apparently more complex enzymic hydrolytic reactions of monosubstituted orthophosphates do not reveal such a multiplicity of reaction mechanisms, at least within the framework of our present fragmentary knowledge. A number of years ago, we set out to determine whether the C—O bond or the P—O bond was cleaved in the enzymically catalyzed reactions of organic phosphates by studying the reactions with O^{18}. The first reactions studied in this manner were the hydrolysis of glucose 1-phosphate by prostatic acid phosphatase and intestinal alkaline phosphatase, respectively (Cohn, '49). The appearance of 1 atom of O^{18} in the inorganic phosphate demonstrated P—O bond cleavage in both reactions, and since not more than 1 atom of O^{18} was found, a quinquevalent phosphorus intermediate was eliminated. These reactions have now been studied with a variety of substrates, the alkaline phosphatase catalysis by Stein and Koshland ('52), the acid phosphatase catalysis by Bunton *et al.* ('57). Bentley ('49) studied the hydrolysis of acetyl phosphate catalyzed by a specific acetyl phosphatase. Koshland and Springhorn ('56) also studied a specific phosphatase, 5′-nucleotidase. In all cases, 1 atom of O^{18} appeared in inorganic phosphate, consistent with the findings with glucose 1-phosphate.

When the same substrate (glucose 1-phosphate) participates in two other reactions (polysaccharide and sucrose phosphorylase), the C—O bond is cleaved, yielding inorganic phosphate and a glycoside (Cohn, '49). In the phosphorylase

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[2] The experimental work described in this paper was done during the tenure of an Established Investigatorship of the American Heart Association.

reactions, the attack is on the carbon of glucose 1-phosphate, and in the phosphatase reactions the attack is on the phosphorus. Thus the phosphatases may be considered as phosphoryl-transferring enzymes and the phosphorylases as glucosyl-transferring enzymes.

An extension of this approach to kinase reactions and other phosphorylase reactions led to the emergence of a pattern. As shown in table 1, there are a number of phosphate substrates that may function as dual donors either of phosphoryl groups (column I) or of the other group such as glycosyl or acyl (column II). The reactions in column I may be regarded as displacement reactions on the phosphorus either by the enzyme or another reactant. Of the reactions listed in column I, direct experimental evidence for P—O cleavage has been obtained only for carbamyl phosphate (Stulberg and Boyer, '54) and 1,3-diphosphoglycerate (Harrison *et al.*, '55; Cohn, '56) but many kinase reactions, pyruvate kinase (Harrison *et al.*, '55), adenylate kinase (Cohn, '56) and hexokinase (Cohn, '56), have been shown directly to involve P—O cleavage. The reactions listed in column II may be regarded as displacement reactions on the carbon by either the enzyme or another reactant. In the reverse direction, they may be considered conversions of inorganic phosphate to organic phosphate, and the oxygen involved in the bridge between carbon and phosphate always arises from inorganic phosphate. In such reactions, in contrast to the class on the left, the formation of a covalently bonded enzyme phosphate intermediate would be excluded.

Let us now examine some known characteristics of these two classes of reaction that limit the type of mechanism that may be postulated. Some of the generalizations deduced from experimental observation are listed in table 2. We note that, unlike nonenzymic hydrolytic reactions, enzymic hydrolyses never occur with C—O cleavage. The hydrolysis of acetyl phosphate by glyceraldehyde 3-phosphate dehydrogenase, described by Park and Koshland ('58) as a C—O cleavage, is only an apparent exception to this generalization since in this reaction it is probably the acyl—enzyme bond, as the authors suggest, rather than

TABLE 1

Types of phosphate substrates with dual function

Phosphoryl group transfer (I)		Substrate	Other group transfer (II)		
Acceptor	Products		Acceptor	Product (+ P_i)	Bond formed
α-Glucose-1-P	Glucose-1, 6-diP + glucose	← α-Glucose-1-P →	Polysaccharide Monosaccharide	Glycoside Disaccharide	C—O C—O
		← Ribose-1-P →	Purine Pyrimidine	Nucleosides	C—N
ADP	ATP + NH_3 + CO_2	← Carbamyl-P →	Ornithine Aspartate	Citrulline Ureidosuccinate	C—N C—N
ADP	ATP + acetate	← Acetyl-P →	CoA	Acetyl CoA	C—S
ADP	ATP + 3-phosphoglycerate	← 1,3-Diphosphoglycerate →	Enzyme	Acyl—enzyme	C—S

TABLE 2

Characteristics of phosphoryl transfer (I) and nonphosphoryl transfer (II) reactions

I (C—O⋮P)	II (C⋮O—P)
1. Only O—P bonds formed[a]	1. O—C, N—C, and S—C bonds formed
2. Mg^{++} or other bivalent cation essential or stimulatory	2. No effect of Mg^{++} or other bivalent cation
3. Specific and nonspecific reactions with H_2O or alcohol as acceptor	3. No reaction with water by this bond cleavage

[a] An exception to this generalization has been reported by Cori *et al.* ('58); namely, the formation of an N—P bond in the phosphorylation of creatine by 1,3-diphosphoglyceric acid.

e C—O bond of acetyl phosphate that is ydrolytically cleaved. The activating fect of Mg^{++} for P—O cleavages and not r C—O cleavages is analogous to Westeimer's finding ('57) that the solvolysis of trabenzyl pyrophosphate is increased bout 1000-fold by 0.02 *M* Ca^{++}. Furtherore, in that case in the absence of the bilent electrophilic cation or an effective ucleophilic reagent, the solvolysis proeeds only by C—O cleavage. Boyer and arrison ('54) and Westheimer ('57) sugested that the function of Mg^{++} in enmic phosphoryl transfer may be to induce creased positive charge on the phosiorus, thus rendering it more susceptible nucleophilic attack.

REACTIONS OF GLUCOSE 1-PHOSPHATE

The reactions of glucose 1-phosphate ill be discussed in detail as an example a substrate that may function as a glysyl donor or a phosphoryl donor. It was iosen for the following reasons: (1) it is e substrate for many specific and nonecific reactions; (2) considerable inrmation has accumulated on both nonnzymic and enzymic reactions that can be pplied to a mechanistic description of its actions; and (3) the available data illustrate the criteria used in determining mechanism.

In reactions of glucose 1-phosphate to form glycosides and inorganic phosphate, the phosphorylase reactions, several experimental criteria exist for the mechanism. Koshland ('54) discussed these criteria in terms of a single displacement mechanism for such reactions in considerable detail including: (1) retention or inversion of configuration, (2) cleavage point, (3) exchange of inorganic phosphate with glucose 1-phosphate in the absence of acceptor, and (4) specificity. That not all glucosyl transfer reactions from glucose 1-phosphate share the same mechanism becomes immediately obvious by applying these criteria (see table 3). Only one criterion—the cleavage point—C—O cleavage, is the same for polysaccharide, sucrose, and maltose phosphorylase. The cleavage points for polysaccharide and sucrose phosphorylase were obtained directly from O^{18} data and that for maltose phosphorylase was inferred from the inversion of configuration from β-glucose 1-phosphate to α-1,4-maltose. The enzymic cleavage of glucose 1-phosphate at the C—O bond may be related to the very high rate of nonenzymic hydrolysis of the neutral species with C—O cleavage, which

TABLE 3

Transglycosyl reactions of glucose 1-phosphate

Phosphorylases	Cleavages	Stereochemistry	Exchange (P ⇄ G-1-P)
Polysaccharide	C—O	Retention of configuration	Negative
Sucrose	C—O	Retention of configuration	Positive
Maltose	C—O	Inversion	Negative

is peculiar to glucose 1-phosphate and does not occur readily with other phosphate esters (Bunton *et al.*, '58). In spite of the identity of cleavage point, polysaccharide and sucrose phosphorylase reactions occur with retention of configuration contrary to the maltose phosphorylase reaction, which occurs with inversion. The exchange of glucose 1-phosphate with inorganic phosphate is catalyzed only by sucrose phosphorylase and not by polysaccharide or maltose phosphorylase. Only the maltose phosphorylase reaction is consistent with a single displacement reaction with the usual "back-side" attack of the acceptor glucose on carbon 1 of the β-glucose 1-phosphate with concomitant inversion upon cleavage of the C—O bond and formation of α-1,4-maltose. The absence of an exchange reaction with this enzyme is consistent with this single-step mechanism. The existence of the exchange reaction of glucose 1-phosphate and inorganic phosphate catalyzed by sucrose phosphorylase brought into prominence the concept of a stable glucosyl enzyme molecule as an intermediate (Doudoroff *et al.*, '47). Koshland ('54) pointed out the theoretical limitations of using the exchange criterion alone for establishing the existence of such an intermediate. In addition to the observation of an exchange reaction in the absence of acceptor, it is necessary, owing to specificity considerations, to establish that phosphate cannot, or at least is highly unlikely to, act at the site of the acceptor molecule and that the exchange reaction also proceeds with retention of configuration. All these conditions are met in the sucrose phosphorylase reaction, and the most plausible formulation for the reaction is a two-step reaction, each a single-displacement reaction on carbon 1 with no net change in configuration. Since the exchange criterion has been used so widely to imply the existence of the stable enzyme intermediates, it might be well to inject a note of caution from the experimental point of view. The validity of this criterion depends on the complete absence of acceptor molecules in the exchange system to eliminate the possibility of the reversal of the over-all reaction.

Polysaccharide phosphorylase does not catalyze an exchange of glucose 1-phosphate with inorganic phosphate, and
inversion occurs. Therefore, it does n
fall mechanistically into the same catego
as either of the other two phosphorylase
Two mechanisms are consistent with t
available data, a single-displacement rea
tion with a "front-side" attack on carbon
or a double-displacement reaction with
unstable glucosyl enzyme intermedia
At present, there are insufficient da
available to distinguish between the tw
mechanisms.

Phosphoryl transfers

Since there have been no mechanis
studies of the reaction listed in table 1 f
glucose 1-phosphate as a phosphoryl don
(the formation of glucose 1,6-diphosphat
(Leloir *et al.*, '49; Sidbury *et al.*, '56),
shall consider the hydrolytic reactions
glucose 1-phosphate as typical phospho
transfers. Both the nonenzymic and e
zymic hydrolyses have been investigate

The nonenzymic hydrolysis of gluco
1-phosphate was studied by Desjobert ('5
and more recently by Bunton *et al.* ('58
The criteria used for establishing the mec
anism have been (1) specificity, (
cleavage point, and (3) molecularity.
a combined study of the *p*H profile a
hydrolysis in H_2O^{18}, Bunton *et al.* ('5
established that the monoanion of gluco
1-phosphate is hydrolyzed via P—O bo
cleavage. This type of bond cleava
seems to be general for the monoani
species, having now been demonstrat
for benzyl (Kumamoto and Westheim
'55), 2-methoxy-1-methyl (Butcher a
Westheimer, '55), glycerol 1- and glyce
2- (Swoboda and Crook, '55), meth
phenyl, *p*-tolyl, *p*-nitrophenyl (Bunton
al., '58) phosphate esters as well as
glucose 1-phosphate. The rates of hydro
sis are not very different for the mono
ion of any of these esters. Bunton *et*
('58) pointed out that it is difficult to sp
ify the precise mechanism of the mo
anion hydrolysis because, for the con
tions under which reaction occurs, th
is no appropriate experimental criter
for molecularity. However, certain rest
tions are imposed on any proposed me
anism by the available data. First,
reaction is limited to those species c

ning an ionized and an un-ionized acid up, i.e., the O^- and —OH groups must present. The necessity for an —OH up is indicated by a ratio in the rates at least 5000 between dimethyl (Me_2 $^-$), which lacks an —OH, and the nomethyl phosphate ($MeHPO_4^-$). The gligible rate of hydrolysis of the dianion PO_4^- of methyl phosphate, which also ks an —OH group, again points to the essity of an —OH in the substrate. e necessity for an O^- group is indicated the fact that the neutral species does undergo this reaction but is hydrolyzed water via a C—O cleavage.

as a model of the Mg^{++}-catalyzed alkaline phosphatase.

The hydrolysis of glucose 1-phosphate catalyzed by prostatic acid phosphatase has many features in common with the nonenzymic hydrolysis of the monoanion species. It occurs with P—O cleavage, is specific for monoesters, and the maximum velocity does not vary from one ester to another. The *p*H of optimum catalytic activity is approximately where the monoanion is the most abundant species. On the basis of variation of K_m with *p*H with *p*-nitrophenyl phosphate as substrate, Bunton *et al.* ('57) suggested that the

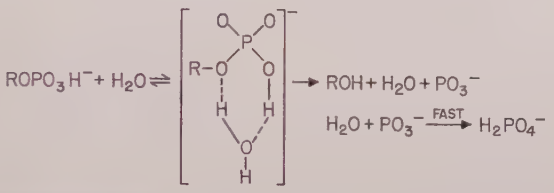

Fig. 1 Mechanism of hydrolysis of monoanion of phosphate monoester proposed by Butcher and Westheimer ('55).

On the basis of the specificity and P—O d cleavage, Butcher and Westheimer 5) formulated the mechanism shown igure 1 for the hydrolysis of the monon species. This mechanism involves formation of a cyclic intermediate med by hydrogen bonding between the noanion and a water molecule followed transfer of the H atom from water to ester oxygen atom. The decomposition he intermediate can occur readily be- se ROH rather than RO^- is formed. e unstable monomeric metaphosphate med is rapidly hydrated to form orthosphate. Other formulations are pose, but all involve cyclic intermediates proton transfer to form ROH.

Butcher and Westheimer ('55) found t, although the dianions of phosphate rs are resistant to hydrolysis, at *p*H a typical monoester, 1-methoxy-2-propyl phosphate is hydrolyzed by $La(OH)_3$ n complete retention of configuration with cleavage of the P—O bond. Esially the same mechanism is postud as for the monoanion with the posi- La^{+++} ion replacing the proton of the noanion. This mechanism is suggested monoanion is linked to the phosphatase surface by two groups, one of which is negatively charged and the other uncharged. It is difficult to evaluate the plausibility of this mechanism on the basis of the data presented. They suggest a modification of Westheimer's mechanism with the hydrogen bonding to the enzyme instead of water and the transfer of a proton from the enzyme to the ester O to form ROH.

Extrapolation from the nonenzymic mechanisms to the enzymic mechanisms presents certain obvious difficulties. In the prostatic acid phosphatase reaction, it is not known which is the reactive ionic species, and unfortunately it is no simple matter to determine it unequivocally. Bunton *et al.* ('57) neglected the role of Mg^{++}, and since P—O cleavage is characteristic of both the monanion pathway and the metal ion-catalyzed pathway of the dianion, the cleavage criterion fails to distinguish between the two. The metal ion-catalyzed reaction of the dianion might well serve as a model for the alkaline phosphatase reaction were it not for the data of Reid and Copenhaver ('57), which indi-

cate that the dianion species is not the reactive substrate for intestinal alkaline phosphatase. Exchange reactions have been investigated and alkaline phosphatase has been found to catalyze a very slow exchange between phosphate and H_2O^{18} (Koshland *et al.*, '54); no such exchange could be observed with prostatic acid phosphatase (Bunton *et al.*, '57). Two specific phosphatases, those hydrolyzing serine phosphate and glucose 6-phosphate have been reported to catalyze an exchange between serine phosphate with serine (Neuhaus and Byrne, '59) and glucose 6-phosphate with glucose (Hass and Byrne, '58). In conclusion, it would seem premature to attempt a formulation of a detailed mechanism of enzymic hydrolysis of orthophosphate esters in view of the gaps in essential information.

REACTIONS OF NUCLEOSIDE TRIPHOSPHATES

For a consideration of phosphoryl transfers, it is most profitable to consider some aspects of bond cleavage of the nucleoside triphosphates, in particular ATP, the most versatile of all phosphoryl donors. Figure 2 illustrates the various points of cleavage

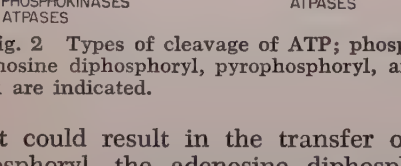

Fig. 2 Types of cleavage of ATP; phosphoryl, adenosine diphosphoryl, pyrophosphoryl, and adenyl are indicated.

that could result in the transfer of the phosphoryl, the adenosine diphosphoryl, the pyrophosphoryl, and the adenyl groups. I should like to emphasize from the outset the symmetry of the phosphoryl and adenosine phosphoryl groups. Often the phosphate groups of ATP are referred to as terminal, middle, and innermost, but it is more helpful for considerations of reaction mechanism to consider them as two terminal groups (although one is substituted) and a third group, the middle one. The most commonly encountered type of reaction of ATP is the cleavage of the unsubstituted terminal phospha group, the so-called kinase reactions. A the kinase reactions whose cleavage poin have been investigated directly with O including hexokinase (Cohn, '56), 3-pho phoglycerate kinase (Harrison *et al.*, '5 Cohn, '56), adenylate kinase (Cohn, '56 and pyruvate kinase (Harrison *et al.*, '55 exhibit a cleavage of the bond between t terminal P and O and may be interpret as a displacement reaction with a nucle philic attack on the terminal P. The h drolytic cleavage of ATP by several d ferent ATPases (Koshland *et al.*, '5 Cohn, '56; Cohn and Meek, '57) to for ADP and orthophosphate has been shov to involve the same cleavage. An ever-i creasing number of reactions involving t cleavage of the substituted terminal grou the adenyl group, are being recognize Boyer *et al.* ('56) demonstrated a cleava between the P of the adenyl moiety and in the adenyl transfer in the acetate ac vation reaction; all members of this cla of reactions probably have the same ty of bond cleavage. These reactions m be described in analogous terms to t phosphokinase reactions as displaceme reactions with a nucleophilic attack on t P of the adenyl moiety.

No reactions have been described which a nucleoside diphosphoryl group transferred. Although a large number compounds of the type uridinediphospha glucose are known, they are formed n by a transfer of a uridine diphospho group from UTP to glucose but by transf of the substituted terminal group of UT the uridyl group to glucose 1-phospha It would seem that the middle P is ve resistant to attack. There are two reactio in which monosubstituted pyrophospha compounds are derived from ATP, t formation of 5′-phosphoribosyl pyroph phate from 5′-phosphoribose and ATP a the formation of thiamine pyrophospha from thiamine and ATP. In the formati of 5′-phosphoribose pyrophosphate it h been shown with P^{32} (Khorana *et al.*, '5 that the middle and terminal P of A are the sources of the pyrophosphate the product. The thiamine pyrophospha formation proceeds in the same way (F sander, '56). However, the question ma

TABLE 4

Hydrolysis of nucleoside triphosphates yielding inorganic pyrophosphate

Substrate	Enzyme source	O^{18} concentration (atom % excess)			
			Pyrophosphate		
		H_2O	Observed	Calculated	
				XP ⋮ O-PP	XP-O ⋮ PP
ATP	Snake venom (Naja naja)	1.22	0.000	0	0.153
		1.30	0.000	0	0.163
dCTP	T2-infected *E. coli*	10.10	0.069	0	1.263

ll remains in figure 2 since the cleavage int, though most likely in the position dicated, has not yet been determined rectly.

We have recently studied the hydrolysis nucleoside triphosphates catalyzed by zymes that yield pyrophosphate and nu-otide to determine the cleavage point, ether it is an attack on the P of the enyl group with cleavage between the of the adenyl group and the bridge oxy-n or an attack on the middle P with avage between it and the bridge oxygen. vo reactions were studied in H_2O^{18}, the drolysis of ATP by cobra venom, which lds AMP and pyrophosphate (Johnson *al.*, '53), and the hydrolysis of deoxy-tosine triphosphate by an enzyme from -infected *Escherichia coli,* which yields rophosphate and deoxycytosine mono-osphate. The substrate and enzyme for e latter reaction were kindly supplied Dr. A. Kornberg and Mr. S. Zimmerman. e absence of O^{18} in the pyrophosphate as own in table 4 demonstrates that both ese hydrolytic enzymes catalyze a cleav-e of the nucleotidyl group rather than e pyrophosphoryl group. The small nount of O^{18} in the pyrophosphate re-ting from the hydrolysis of dCTP is e result of a contamination of the pyro-osphate with orthophosphate produced inorganic pyrophosphatase in the en-me preparation.

The cobra venom preparation, in addi-n to the ATPase, has a very active 5′ cleotidase and some inorganic pyrophos-atase. The products of hydrolysis e therefore adenosine, orthophosphate, d pyrophosphate. The orthophosphate formed should contain 2 atoms of O^{18}, one being introduced from H_2O^{18} by the ATPase and the second by the 5′ nucleoti-dase. However, some of the orthophos-phate arises from the action of inorganic pyrophosphatase with 0.5 atom of O^{18} per orthophosphate molecule formed by this reaction. From the ratio of orthophosphate to pyrophosphate in the reaction products and on the assumption that the activity of the 5′ nucleotidase was sufficiently high to hydrolyze all the AMP formed by ATPase action, the maximum number of O^{18} atoms anticipated in the orthophosphate was 1.5. The observed value in the first experiment was 1.2. Thus it was demonstrated that the hydrolysis of nucleoside triphosphates to yield nucleotide and pyrophosphate may be considered nucleotidyl transfer proceed-ing by attack on the substituted terminal P rather than on the middle P.

The similarity of the two terminal phos-phorus atoms is strikingly demonstrated by the nuclear magnetic resonance spec-trum of ATP (fig. 3). In this type of spectroscopy, the shift in resonance fre-quency of any given P atom in a molecule, relative to its values for an isolated P atom, depends on the electronic environ-ment of the P atom within the molecule. In the upper curve, which is the spectrum of the disodium salt of ATP, the peak at the far right is the middle P atom and the two terminal P's are superimposed upon each other. The middle P is identified from previous spectra of inorganic poly-phosphates (van Wazer *et al.*, '56) and by higher-resolution spectra, which show that the middle P is split into a symmetrical triplet owing to interaction with the two

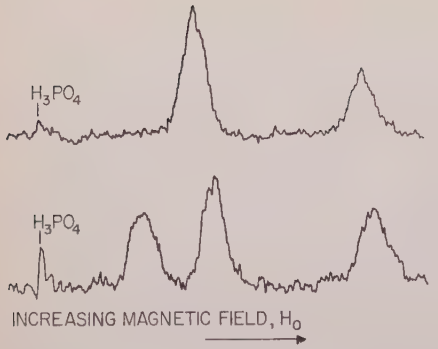

Fig. 3 The upper curve is the nuclear magnetic resonance spectrum of the disodium salt of ATP; the lower curve is the spectrum of the tetramethylammonium salt at *p*H 8.5.

neighboring P atoms. As the *p*H of the solution is increased, the terminal P's separate because of ionization of the last —OH group. The lower curve shows the spectrum of the tetramethylammonium salt at *p*H 8.5. At higher resolution, the splitting of the terminal P's into doublets by interaction with a single neighboring P becomes visible. Even at *p*H 8.5, it is obvious from the curves that the electronic environments of the two terminal P's are more similar than the electronic environment of the middle P.

In table 5, known types of phosphoryl and nucleoside phosphoryl transfer from nucleoside triphosphates are listed. In the former, nucleoside diphosphate is always a product and in the latter, pyrophosphate is always a product. The list of reactions in this table is by no means complete bu includes many of the most important bio synthetic reactions that occur in the cell More than 40 alcohols can act as acceptors of phosphoryl groups to form phosphate monoesters. There is only one example o an alcohol acceptor for the nucleoside phos phoryl group to form phosphate diesters namely, DNA in the DNA polymerase re action (Bessman *et al.*, '58), a fairly com plex reaction that may be formulated a follows:

$$n(\text{dTPPP} + \text{dCPPP} + \text{dGPPP} + \text{APPP}) + \text{DNA} \leftrightarrows \text{DNA—(TP—CP—GP—AP)}_n + 4\,(n)\text{PP}$$

Both types of transfer lead to carboxyli anhydrides; only phosphoryl transfer i listed as yielding N—P bonds. It can b seen that participation by either end grou of nucleoside triphosphates occurs wit similar frequency. Perhaps acetate activa tion as shown in figure 4 best illustrate the parallelism of the alternate reactio paths. On the right, ATP cleaves at on end to react with acetate to yield acet phosphate and ADP, reaction (1); on th left is the parallel reaction with a cleavag at the other end to yield acetyl adenyla and pyrophosphate. Either one of the ac phosphates can then react by C—O clea age with CoA to yield acetyl *S*-CoA an orthophosphate and adenosine monopho phate, respectively. It should be noted th both reactions (1) and (2) require Mg^+ but neither (3) nor (4) are affected b

TABLE 5

Transfer of orthophosphoryl and nucleoside phosphoryl groups

Bond formed	Transfer of $-\overset{\overset{O}{\|}}{\underset{\underset{O^-}{\|}}{P}}-O^-$ Acceptor	Transfer of $-\overset{\overset{O}{\|}}{\underset{\underset{O^-}{\|}}{P}}-O-X$ Acceptor
Ester	Alcohols (> 40)	DNA
Carboxylic anhydride	3-P-glycerate, acetate, aspartate, carbamate	Fatty acids, amino acids
Phosphoamidate	Creatine, arginine	—
Phosphoric anhydride	Nucleoside monophosphate, nucleoside diphosphate, inorganic polyphosphate	Nicotinamide mononucleotide, riboflavin phosphate, phosphopantotheine, hexose phosphates, choline phosphate
Other	Enol pyruvate	Sulfuric acid, luciferin

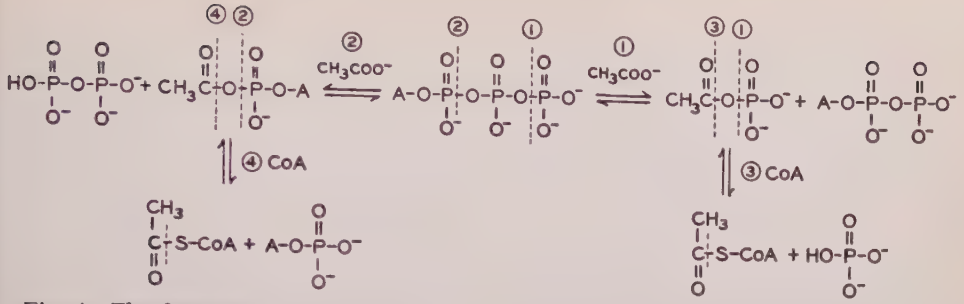

Fig. 4 The formation of acetyl *S*-CoA via acetyl phosphate, reactions (1) and (3) and via acetyl adenylate, reactions (2) and (4).

g^{++}. The mechanisms of the reactions the two sequences are not identical, wever, since acetyl adenylate has never en isolated as a reaction product and presumably enzyme bound (Berg, '56). rthermore, both reactions (2) and (4) the adenylate sequence are catalyzed a single enzyme, contrary to the phos- ate sequence in which reactions (1) and) are catalyzed by two distinct enzymes. The question has often arisen: Why is that some enzymes catalyze the cleavage orthophosphate from ATP and others talyze the cleavage of pyrophosphate om ATP? The answer is not yet known, t at least we now know how to pose the estion properly: Why is it that some zymes cleave a phosphoryl group from TP leaving a substituted pyrophosphate DP), whereas others cleave a substituted osphoryl group, the adenyl group from TP, leaving inorganic pyrophosphate? Although the symmetry of ATP reactions s been stressed from the mechanistic int of view, dissymmetry becomes apparent if we examine the metabolic interrelations of the formation and utilization of ATP by the two types of sequences indicated. The function of ATP in transferring phosphate was defined earlier as a means of transferring energy from one metabolic process to another. The over-all reactions of the cell involving phosphate transfer are represented schematically in figure 5.

The formation of ATP via oxidative phosphorylation and glycolysis occurs through one sequence involving orthophosphate and ADP. Although utilization of ATP for biosynthetic reactions occurs by cleavage of ATP to yield ADP and orthophosphate, a large portion of the biosynthetic pathways occur through the parallel pathway to yield pyrophosphate and AMP. The latter products may be converted to orthophosphate and ADP by two ubiquitous enzymes, inorganic pyrophosphatase and adenylate kinase, respectively. The inorganic pyrophosphate hydrolysis is practically irreversible and favors biosynthetic

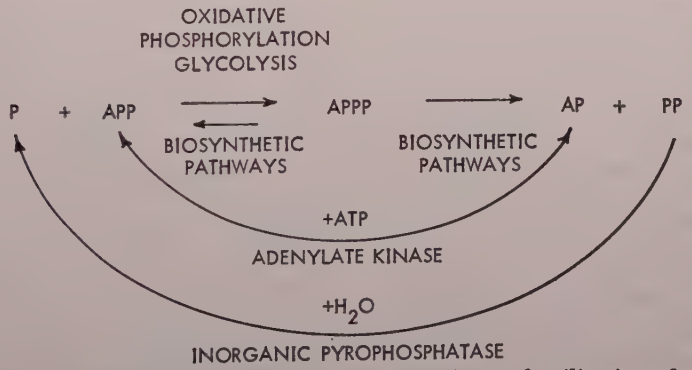

Fig. 5 Metabolic interrelations in the formation and utilization of ATP.

reactions involving pyrophosphate formation and also replenishes the supply of inorganic orthophosphate. The cycle is now complete, and inorganic phosphate is once again available for formation of ATP through coupling with energy-yielding reactions.

ACKNOWLEDGMENT

I wish to thank Dr. J. N. Shoolery and Dr. L. Johnson of Varian Associates for the use of their instrument and their aid in obtaining the nuclear magnetic resonance spectra.

OPEN DISCUSSION

BRESLOW[3]: What do you think is the significance of the NMR spectrum?

M. COHN: The chemical shift of the middle phosphorus of ATP is, in the usual units of NMR spectra, 21 parts per million, and this is a large shift compared to most other phosphate compounds. I must add that this work on the NMR spectra has just started, and we do not have enough spectra yet to make any kind of systematic interpretation. We had hoped that we would show some very dramatic effect by magnesium chelation. This hope was not realized but, interestingly enough, the magnesium has more effect on the middle phosphorus than on either of the terminal phosphorus atoms.

We have also looked at the proton spectra of ATP to see if they were affected by metal chelation; of the three metal ions —magnesium, calcium, and zinc—that we have examined, only zinc has a large effect. It shows definite interaction with one of the protons on the doubly bonded carbon of the adenine ring.

BOWMAN[4]: The first point I wish to make concerns the pyrophosphatase activity present in snake venoms. I am convinced that this is the same enzyme as the phosphodiesterase. All preparations I have worked with split ATP to AMP and pyrophosphate. They also split DPN into AMP and nicotinamide nucleotide. This fits very nicely with the exonuclease activity of the diesterase and shows that the active site of the enzyme has an affinity for the 5′ nucleotide part present in the end of the DNA molecule and in ATP and DPN.

My second point concerns Dr. Khorana paper and the selective influence of t metal ions during the digestion of DN It is known that both the pancreatic DNa I and the venom diesterase have a marke ly different effect on DNA if you contr the metal ion carefully; that is, by havi a bottom level of EDTA and then addi an excess of either calcium or magnesiu Both enzymes show different types of ki etic curves with the different metals; th may be an approach to obtain a limit and well-defined digestion of DNA. I al think that this provides an explanati for the very interesting result Dr. Khora showed with the digestion of a pentan cleotide. The two types of bonds split m be caused by the presence of both calciu and magnesium.

COHN: I should like to say a few wor about the effects of metal ions. Even the nonenzymic reaction it is very difficu to understand just how the metal io function because of the amazing speci city. For example, I have been talki glibly about magnesium increasing t positive charge on phosphorus and maki it more susceptible to nucleophilic attac However, in the nonenzymic hydrolysis ATP, the magnesium chelate of ATP very resistant to hydrolysis. On the oth hand, the barium salt of ATP is very rea ily hydrolyzed. These experimental fac are not easily interpreted. Certainly t question of the effect of various metal io on enzymic ATPases cannot be understo simply on the basis of metal chelation substrates. I have looked at the nucle magnetic resonance spectra of both c cium and magnesium ATP and have c served no difference. It becomes necessa to invoke the interaction of the metal i with the enzyme rather than substra alone in order to explain the difference behavior of Ca^{++} and Mg^{++}.

TODD[5]: This problem of metal ions a their significance is very complex a as Dr. Cohn has said, it is not at all cle just what they are doing. Different io have been observed to show different effects, even in nonenzymic systems, a

[3] Ronald Breslow, Columbia University.
[4] H. G. Bowman, The Rockefeller Institute.
[5] Alexander Todd, University Chemical Labo tory, Cambridge, England.

ey are at times difficult to understand. I not think you could explain away the zyme reactions simply on the basis of tal ion effects.

Perhaps I might make a comment about e different ways in which ATP splits. e could, I think, readily imagine the split- g of the molecule in any of the places ere it normally occurs if we are pre- red to allow localization of the protons the partially ionized molecule. For stance, if in the mono-ion of ATP the ic charge was located on the terminal osphorus, then ATP in that form should lit so as to transfer phosphate. In other rds, it would be capable of breaking up yield monomeric metaphosphate, which uld be a phosphorylating agent, and the n of ADP. Similarly, if the monoanion ATP carried its charge on the phos- orus next to the adenosine part of the olecule, then we would expect by a simi- mechanism to transfer adenylic acid d to throw out pyrophosphate as an ion. I sometimes think that this kind effect is an important feature of enzyme actions and that it is worth considering ether the protein component of the en- me may not have an effect of this type the charge localization. Metal ions ay or may not be involved in this con- l of the ionization.

Incidentally, is it possible that the real nction of the middle phosphorus in ATP simply to provide, by hydrogen bonding, easy method of controlling this possi- lity of ionization of P_1 and P_3? If this is , then we would expect to find what is tually observed, namely, that the middle osphorus of ATP does not turn up as a osphorylating center. Only P_1 and P_3 n function in this respect.

I was very interested in your NMR ex- riments because we have begun to do me experiments on rather similar lines. ur particular aim was to find out whether e could, under different conditions, de- ct any location of ionization of individual osphorus atoms in connection with the ove-mentioned ideas on the possible ef- ct of enzyme proteins.

You mentioned the question of CO and O fission in connection with, say, glucose phosphate. I would prefer to use slightly fferent words and call it the question of alkylation or phosphorylation. In the laboratory, a phosphate can obviously be either an alkylating or a phosphorylating agent; and anything that is done to increase the electrophilic character of the phosphorus atom in the compound will, at the same time, have a similar effect on the α-carbon atom of an esterifying group. These effects cannot be wholly separated and, in fact, it turns out that whether a phosphate will react as an alkylating or a phosphorylating agent depends primarily on the nature of the esterifying group. For instance, if allyl or benzyl phosphate is used, C—C bonds can be formed very easily, and this is something that depends on the particular characteristics of these groups. Glucose 1-phosphate has some of the characteristics of an alkylating phosphate and, indeed, functions as such in a number of enzyme systems. How far the difference between the two types of enzymes can be accounted for by the effect of the protein component on the anionic stability of the phosphate group is, of course, difficult to assess quantitatively. Perhaps you would like to say something on this Dr. Khorana?

KHORANA[6]: I just wanted to make three points. First, will ATP cause phosphorylation or pyrophosphorylation or, alternatively, undergo phosphorolysis or pyrophosphorolysis? This is really very speculative, but the argument can be developed further that the three phosphates forming the chain may assume a configuration, perhaps by chelation with a metal, such that the enzyme may approach at two of the phosphorus atoms, and, depending on the side or the face of this triphosphate structure that the enzyme attaches itself to, it may be phosphorolysis or pyrophosphorolysis. Second, it is interesting to note from what we learned with ribose phosphates that all enzymes of nucleotide metabolism, namely, ribo- and deoxyribonucleoside phosphorylases and nucleotide pyrophosphorylases, involve an inversion at the glycosyl bond (C_1 of ribose ring). This is a unique situation. On the other hand, with di- and polysaccharide phosphorylases, both types of enzymes are known: those that bring about inversion (maltose

[6] H. G. Khorana, British Columbia Research Council, University of British Columbia.

phosphorylase) and those that cause retention of configuration (e.g., sucrose phosphorylase).

Finally, it is interesting that in biology, we find with acyl phosphates both acyl and phosphoryl transfer. Chemically, usually and at least kinetically, the attack is favored on the carbonyl carbon of acyl phosphates. But if we have a reaction in which we take 1 mole of an anhydride, say acetic anhydride, and 2 moles of phosphate ester, it is interesting to note that, although the exchange reactions involving attack at the carbonyl carbon are rapid, the slower attack on the phosphorus atom eventually leads to the formation of the thermodynamically stable pyrophosphate. This is really analogous to the recent enzymic findings on the virtually quantitative formation of ATP from inorganic pyrophosphate and acyl and aminoacyl adenylates.

TODD: The results obtained with the acyl phosphate are, I think, in accordance with what one would expect. If we merely looked at the matter from the point of view of acid strength or ionic stability, attack should be primarily on phosphorus. I think that the reason phosphorus is not actually the primary point of attack is that the carbonyl group of the acyl portion is open to an additive attack, whereas with phosphorus no addition to the P=O occurs, and therefore one of the groups attached to the phosphorus must be expelled as an anion before the nucleophile can actually attach itself. But of course, in such a case a 100% attack on one center and none on the other would not be expected, and so I think that over a period you would get some of the phosphate product, as Dr. Khorana observed.

COHN: I should like to ask a question of both you and Dr. Khorana. Has the particular ionic species that reacts been determined in the case of acyl phosphate? Do metal ions have any effect on acyl versus phosphate transfer in that case?

TODD: I do not know whether Dr. Khorana has any answer to these points. As far as I know the first has not been studied in the simple acyl phosphates. I do not think, either, that much is known about the effect of metal ions. There has, of course, been some study of the effect of varying the solvent; clearly change of solvent will affect matters in the phospha anhydrides just as it does in the carboxyl anhydrides, although acetyl phosphate distinct from its esters has not been muc studied, even from this point of view. think that is correct.

KHORANA: Yes.

TODD: There has not been much on th simple acetyl phosphate itself.

BRUICE[7]: Do you think that it might b a good idea to look toward the reaction uncharged nucleophiles with the pho phate ester dianion since this species electrostatically shielded from hydroxid ion attack? I realize that, in the case phosphate esters, the nucleophilic series inverted as compared, say, with the ca boxylic esters, but have you looked at th *p*H profile for the reaction of a neutr nucleophile with a phosphate monoester see if the dianion is attacked?

COHN: I have not. You are quite righ that it is obvious from the *p*H profile the straight hydrolytic reaction that the d anions of phosphate monoesters are ver resistant and obviously there is no OH attack on them. I do not know of an work on simple monophosphate esters wit other attacking groups. There is the wor of Westheimer on tetrabenzyl pyropho phate that shows a strong nucleophilic r agent is required. If a weak nucleophil reagent attacks then C—O cleavage result

BRUICE: What does the *p*H profile these things look like? Do these nucle philes attack the dianion?

COHN: I do not remember what th *p*H profile was in that case. General speaking, for the simple monoesters orthophosphoric acid, the profile is th same in almost all cases. There is a max mum at *p*H 4, which drops off on eith side, and then when the conjugate ac forms in highly acid solution, the ra increases again. One exception is gluco 1-phosphate; the neutral species of th ester reacts very rapidly. This is peculi to glucose 1-phosphate and leads to C— cleavage. This is ascribed to the peculia ity of the pyranoside ring, and it is plau ble by analogy with other reactions of th pyranosides; but in most simple mon esters the neutral species is very unrea

[7] T. C. Bruice, The Johns Hopkins School Medicine.

ve. The conjugate acid is fairly reactive
ıd the monoanion species is the most
active.

BRUICE: Since halides substituted in the
position of glucose undergo $S_{N}1$ reactions
uld it be possible that in the case of glu-
se 1-phosphate some type of $S_{N}1$ reaction
curs at the enzyme surface and then the
cipient carbonium ion is captured?

COHN: Yes, that is actually the sug-
stion that is made by Bunton and his
workers for the hydrolysis of the neutral
ecies of glucose 1-phosphate that, they
ıint out, is unique for this phosphate
ter.

BRUICE: Can you see how an $S_{N}1$ reac-
ın could occur at the active site?

COHN: There are too many imponder-
ıles in enzymic reactions to say at this
ıment whether one type of displacement
more likely than another. We do not
ıow enough about the nature of the at-
cking group and other factors. All I
ıve tried to do is to make some empirical
neralizations of the available informa-
ın, which limits the mechanism one can
stulate. I do not feel that any of us are
t in a position to postulate a unique me-
anism because, even in the nonenzymic
actions, no unique mechanism has yet
en determined for the hydrolysis of the
ınoanions of phosphate monoesters.

TODD: We have just heard that the
ınoanion goes fastest in the hydrolysis.
has been suggested that the difficulty
ıen the dianion is used is that there is a
ielding effect from the negative charge.
we were dealing with nucleophilic at-
ck, surely the charge on the monoanion
ıuld be enough to give a similar shielding
ect.

BRUICE: But the hydrolysis of the mo-
anion occurs *via* an intramolecular elim-
ation or *via* reaction with neutral water.
does not occur by nucleophilic attack of
ıegative species.

COHN: The mechanism that is sug-
sted is the hydrogen bonding with the
ter molecule. I believe that Westheimer
es not consider the negative charge in
formulation, but the Bunton group
s, and they have modified the inter-
diate slightly to take into consideration
negative charge, but, nevertheless, they
also suggested hydrogen bonding with water. An —OH and an O^- are essential in the reactive species.

TODD: The O^- will certainly make a considerable difference to the strength of the P—O bond.

STROMINGER[8]: We were interested in preparing some acetylglucosamine 1-phosphate and the simplest way to do it seemed to be to hydrolyze UDP-acetylglucosamine in alkali. Cahib and Leloir reported that, if UDPAG was heated in $Ba(OH)_2$, UMP and AG-1-P were formed. However, we used NaOH and found almost no hydrolysis. With $Ba(OH)_2$ under the same conditions, UMP and AG-1-P were formed.

Dr. David Lipkin has been studying the base-catalyzed hydrolysis of ATP and has found, although I do not know the details, that the products of hydrolysis of ATP in base also depend on the cation present. I wonder if studies of the basic hydrolysis of various nucleotides, in which various cations are used, would not yield some interesting information on what the effects of metals might be. I also wonder if any chemist would like to provide an explanation for the different effects of different cations on the hydrolysis of nucleotides.

TODD: All I can say about that is that this kind of effect has often been observed in the nucleotide field and elsewhere among the phosphates, and I still have not found a chemist who can give me a watertight explanation for it.

KOSHLAND[9]: I have enjoyed Dr. Cohn's talk very much and think she has done an excellent job of illustrating the multiplicity of pathways available in phosphate transfers, both nonenzymically and enzymically. The phosphate group can act as a nucleophile, in which case the electrons on a phosphate oxygen are the point of reaction, or it can act as a phosphoryl donor, in which case the phosphorus atom is itself attacked by a nucleophilic group. Because of the high negative charge of the phosphate group, positive ions can have great influence. They can influence the binding to enzyme, they can lower the

[8] Jack Strominger, Washington University, St. Louis.

[9] D. E. Koshland, Jr., Brookhaven National Laboratory.

electrostatic repulsion between reacting groups, and they can act as Lewis acids to aid the departure of a leaving group. Their presence, therefore, can make a reaction go at high velocity which would be otherwise undetectable. Moreover, the precise location of these positive ions can determine the course of the reaction, e.g., the innermost versus the terminal phosphate of ATP, and thus allow further control of the biological pathway. Dr. Cohn's paper emphasizes both the necessity for caution in assigning a specific mechanism to an individual enzyme and the important biological value of this multiplicity.

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rticipation of Acyl—CoA in Carbon ain Biosynthesis

FEODOR LYNEN
Max-Planck-Institut für Zellchemie and Chemisches Laboratorium der Universität, Institut für Biochemie, München, Germany

The synthesis of natural products pre-
its to the chemist a challenge that is
ficult yet fascinating. Fortunately,
ough the years numerous techniques for
anic synthesis have become available.
e growing list of complex alkaloids,
roids, antibiotics, and even macromole-
es that have been synthesized bear wit-
ss to the elegance and precision of these
hniques. For the biochemist, each new
vance in the structure of naturally oc-
rring substances is an opportunity to
n further insight into the integrated
thetic process that is the living or-
nism.

The organism itself is, in a sense, under
reater handicap than the chemist faced
h the problem of assembling a complex
lecule: it cannot thumb through Beil-
in for each new task and, if it could,
uld be dismayed at the relatively limited
mber of syntheses possible in an aque-
medium at ambient temperatures.
this purpose, the cell has at its disposal
cient enzymes of a high order of speci-
ty that the organic chemist does not
have. It is the further realization that
se complex molecules are made in the
from simple chemical building blocks
t draws our attention to the underlying
chemical mechanisms by which the
-C bond is formed biologically. Two
h reactions, aldol condensation and
loin condensation, have long been
own. These reactions are discussed by
L. Horecker (this Symposium). A third
ction, and the one with which my paper
l deal mainly, is the formation of the
-C bond at the expense of an acyl—CoA
nd. There is also a fourth type of re-
ion that involves C alkylation. This re-
ion mechanism was discovered in 1958
ring investigations on the biosynthesis of terpenes and could not therefore be considered in time for this symposium. I would like first to discuss the latter mechanism briefly.

In studies on the enzymic conversion of mevalonic acid to squalene by yeast extracts, a new intermediate compound produced from mevalonic acid and ATP (Bloch, '59; Lynen, '59) was discovered. This new compound could be converted to squalene in the presence of TPNH without further addition of ATP. Analytic and synthetic studies proved that the new compound was Δ^3-isopentenyl pyrophosphate (Chaykin *et al.*, '58; Lynen, Eggerer, *et al.*, '58). Isopentenyl pyrophosphate can be considered as a disguised isoprene molecule (fig. 1). It might be supposed that, after release of the pyrophosphate moiety, the remainder of the molecule would participate in the mechanism for terpene formation postulated by Rilling *et al.* ('58). However, we observed, in a mixture of soluble enzymes from yeast, that squalene biosynthesis proceeded via the sesquiterpene, farnesyl pyrophosphate (Lynen, Eggerer, *et al.*, '58). The isolation of this compound spoke against a mechanism involving polymerization of isoprene. Further insight into the mechanism of this biosynthetic pathway was made possible by experiments of Agranoff *et al.* ('59) in our laboratory. They succeeded in fractionating an enzyme from autolyzates of baker's yeast that catalyzes the isomerization of isopentenyl pyrosphosphate by migration of the double bond to form γ, γ-dimethylallyl pyrophosphate (fig. 2). The newly formed dimethylallyl pyrophosphate possesses the allylic configuration particularly effective in the formation of the carbonium ion. The carbonium ion then attacks the

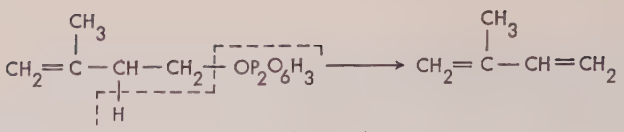


Figure 1

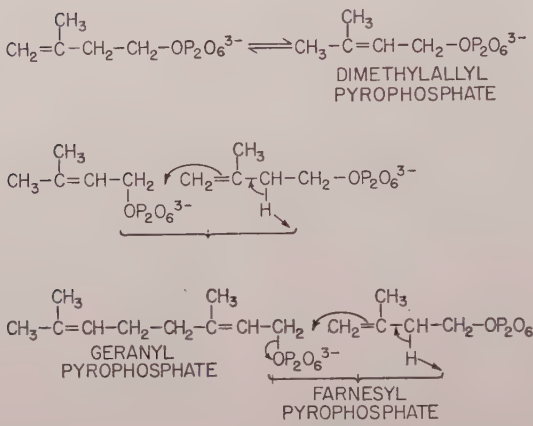


Fig. 2 Mechanism of C—C bond formation in terpene biosynthesis.

reactive double bond of isopentenyl pyrophosphate in an alkylation reaction to form the new C—C bond. The product, after loss of a proton, is geranyl pyrophosphate —another allylic derivative capable of reacting again with isopentenyl pyrophosphate. In fact, we have found that, in yeast extracts, the condensation does not stop at farnesyl pyrophosphate but continues on to the diterpene, geranyl-geranyl pyrophosphate (U. Henning, unpublished data).

The discovery that the vitamins K_2 and the various ubiquinones, or coenzymes Q, possess terpene side chains with up to 50 carbon atoms (Wolf *et al.*, '58; Morton *et al.*, '58; Gloor *et al.*, '58; Isler, '59) leads us to speculate that di-, tri-, or pentaterpenyl pyrophosphates are formed by the foregoing process and finally alkylate methylnaphthohydroquinone or 2,3-dimethoxy-5-methyl-benzohydroquinone by the familiar principles of aromatic substitution (fig. 3). Increased incorporation of 2-methyl-1,4-naphthoquinone into vitamin K upon addition of mevalonic acid in chicken liver homogenates was reported by Martius and Esser ('59). Gloor and Wiss ('58)

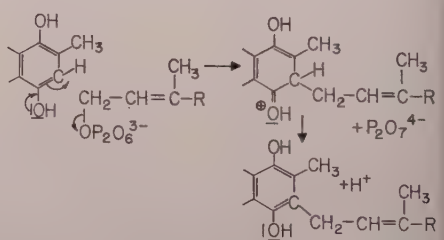
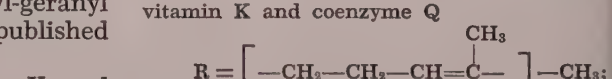


Fig. 3 Possible mechanism of formation vitamin K and coenzyme Q



$n = 3, 4, 5, 6, 7, 8,$ or 9.

demonstrated the biosynthetic incorpo tion of mevalonic acid into ubiquinone

After this brief diversion, I would like return to the main theme of my discussi the participation of acyl—CoA in carb chain biosynthesis. The study of th reactions goes back to the discovery of thioester (or acyl—mercaptan) bond acetyl—CoA, in which the sulfhyd group was identified as the functio group of the coenzyme (Lynen *et al.*, '5 CoA functions in metabolism as an a carrier according to the scheme in figu (Lynen, '52–'53).

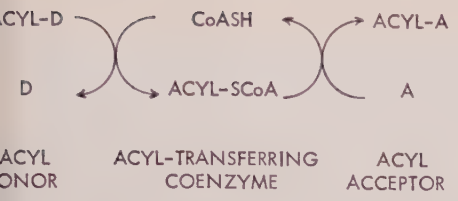


Figure 4

hrough the formation of acyl mercap-
s with CoA, acetic and other carboxylic
ls are transformed into an activated
n in which they can participate in vari-
important metabolic reactions. This
omes understandable when we consider
—SH group of the thioester as a weak
l and the acyl—mercaptan as an an-
ride of a carboxylic acid and substi-
d hydrogen sulfide. These compounds
thus be compared to the "energy-rich"
ydrides of phosphoric acid, such as the
l phosphates, particularly since they
sess high energies of formation. A
ct estimation of bond energy of acyl—
is made by measuring the equilibrium
stants of reversible enzymic reactions
which a thioester bond is formed or
upted. Such studies of citrate conden-
on (Stern *et al.*, '52) or of the CoA-
endent acetaldehyde dehydrogenase
n *Clostridium kluyveri* (Burton and
dtman, '53) give values for the free
rgy of hydrolysis of the acyl—mercap-
bond of $\Delta F^\circ = -7.65$ to -8.25 kcal/
e (Burton, '55). The physiological
ue of such energy-rich compounds is un-
stood when we consider that they are
ble at the cellular range of temperature
pH and become reactive only in the
sence of specific enzymes (Lynen *et al.*,
). Further appreciation of the special

TABLE 1

parison of elements of biological interest (Pauling, '48)

ent	Ionic radius	Nuclear charge	Electro-negativity
gen	1.40 (O^{2-})	8	3.5
ur	1.84 (S^{2-})	16	2.5
on	2.60 (C^{4-})	6	2.5
ogen	1.71 (N^{3-})	7	3.0

properties of thioesters may be derived from a comparison with oxygen esters. First, let us examine some comparisons of sulfur and oxygen (table 1). The larger ionic radius and the relatively higher charge of the sulfur nucleus allow the hydrosulfide ion to dissociate its proton more easily than can the hydroxyl. The —SH bond is therefore more polar, a fact that is not altered by substitution. The higher charge density of sulfur is responsible for one of the principal properties of divalent sulfur — its little tendency to form a double bond (Baddiley, '50), hence its little tendency to participate in resonance. The electronegativity of carbon and sulfur is the same (table 1); therefore, the dipole moment of a symmetrically substituted C—S bond is small but easily polarized by unsymmetrical substitution. This occurs when a mercaptan reacts with a carboxylate ion to form a thioester. The partially polarized carbonyl carbon obtains electrons from the negatively charged oxygen, whereas the electrons of the C—S bond are polarized in the direction of the sulfur. Equalization of this polarization is not possible because the divalent sulfur resists double bond formation. The result is an increased positivity of the carbonyl carbon (fig. 5); i.e., an increased ketone

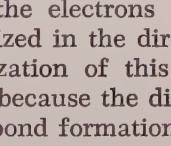


Fig. 5 Polarized state of acyl—CoA.

effect as is found in acid chlorides and as is manifested spectrophotometrically by the absorption at 235 mμ (Sjöberg, '42; Lynen, '53).

This concept of acyl—CoA permits a unified visualization of both the "head" and the "tail" activations so named by Lipmann ('48–'49). In the "head activation" type of reaction, nucleophilic substituents (Lewis bases) attack the positively induced carboxyl carbon and CoA is split off simultaneously. These substituents are shown in figure 6 in order of increasing reversibility of reaction. Here are the al-

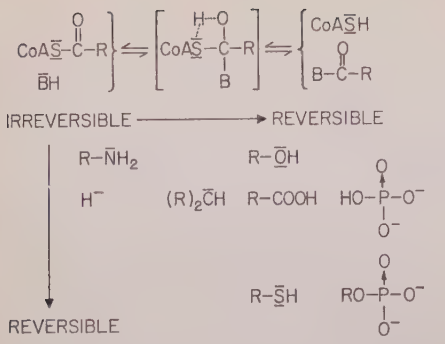


Fig. 6 Nucleophilic substitution of the CO-*S* group.

most irreversible acylations of amines and alcohols, the transacetylations and the thiokinase reactions. Of the various nucleophilic substituents, only the carbanion is pertinent for the formation of carbon chains. By the addition of a carbanion to the acyl—mercaptan bond, a keto acid is produced by a Claisen-type condensation. In the formation of δ-aminolevulinic acid, glycine is condensed with succinyl—CoA (Layer *et al.*, '58; Kikuchi *et al.*, '58). The reactivity of the methylene group of glycine is enhanced by Schiff base formation with pyridoxal phosphate (O=CH—R). This configuration permits the dissociation of a proton with the simultaneous formation of a carbanion that is necessary for the acylation reaction (fig. 7).

In the thiolase-catalyzed reactions leading to the formation of β-keto acids (Lynen *et al.*, '52; Ochoa, '54; Goldman, '5 acetyl—CoA participates as a nucleoph reactant. The two CoA-activated a groups react together in the manner sho in figure 8 (Lynen, '53).

The coenzyme has in this instance functions. One molecule of acetyl—C is the nucleophilic agent and acetate ceptor and the other is the electroph acylating donor. Therefore, the thiol reaction occupies a position between acylation and the condensation reactio

In the condensation reactions we dealing with "tail activation." It is ag a result of a positive charge on the bonyl carbon (see fig. 5). This posit charge effects the polarization of C—H bond with concomitant release a proton and formation of the re nance-stabilized carbanion (fig. 9). rect demonstration of the effect of acyl—mercaptan bond on the α-met group can be observed by potentiome titration of derivatives of acetoacetic a By this technique, the *pK* values of enol dissociation can be measured (ta 2). The differences observed are a fu tion of the substituent on the carboxyl bon. Contrary to the findings of St ('56), the nature of the thiol compon does not appear to be important, as is s by comparing acetoacetyl—CoA with ac acetyl-*N*-acetyl cysteamine (Grassl, '5 This same acidifying effect must also cur in derivatives of acetic acid. Howe

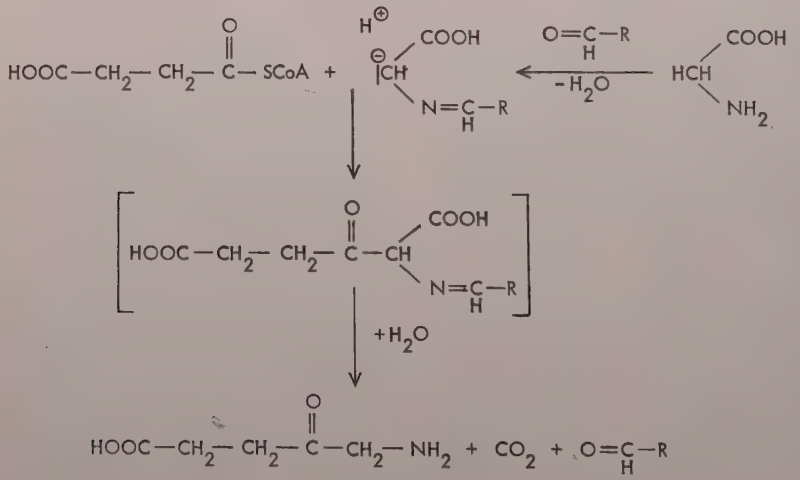


Figure 7

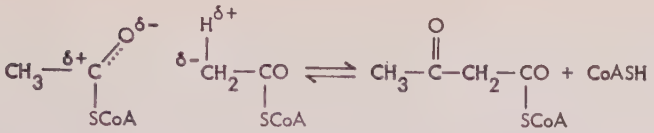


Figure 8

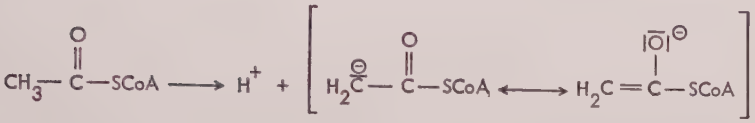


Figure 9

TABLE 2

Enol dissociation of free and bound acetoacetic acid (Lynen, '53)

$CH_3—CO—CH_2—CO—R \rightleftharpoons [CH_3—CO—\ddot{C}H—CO—R]^- + H^+$

Compound	pK'
$CH_3—CO—CH_2—COO^-$	12.70
$CH_3—CO—CH_2—CO—OC_2H_5$	10.70
$CH_3—CO—CH_2—CO—S—CH_2—CH_2—NH—COCH_3$	8.65

he studies of Marcus and Vennesland) have borne out, the hydrogen atoms ie α-methyl group of acyl—CoA do not ome sufficiently acid to exchange with y water, even in the presence of con-ing enzyme. We must therefore con-e that this type of reaction occurs only ng interaction with the reactant in a erted process (Lynen, '58a).

Ve may include the following reactions lectrophilic substitutions on the α-car-of acyl—mercaptans leading to synthe-f the C—C bond: (1) the previously tioned ester condensation with acyl— to form β-ketoacyl—CoA; (2) the tions with keto compounds that are parable to aldol condensation, such as e that lead to citrate, malate, and G—CoA (β-hydroxy-β-methylglutaryl—); (3) the carboxylation reactions of yl—CoA, propionyl—CoA and β-meth-otonyl—CoA involving the participa-of "active CO_2."

ese three types of reactions are sche-cally represented in figure 10. In dis-ing them, I would like first to discuss importance of the ester condensation e biosynthesis of fatty acids. The dif-ty in explaining biological carbon n formation by this reaction lies in the fact that the equilibrium of the thiolase reaction

$$2\ \text{Acetyl—CoA} \rightleftharpoons \text{acetoacetyl—CoA} + \text{CoA}$$

lies far in the direction of splitting. By spectrophotometric techniques, it was possible to establish that, at pH 7,

$$K_{eq} = \frac{[\text{acetoacetyl—CoA}] \times [\text{CoA}]}{[\text{acetyl—CoA}]^2} = 1.6 \times 10^{-5}$$

$$(\triangle F^\circ = +6600\ \text{cal/mole}(28^\circ\text{C.})$$

(Lynen and Decker, '57), which means that at equilibrium, for each 1000 molecules of acetyl—CoA present, there can be only 4 molecules of acetoacetyl—CoA or even less if there is free CoA present. Nevertheless, the synthesis of carbon chains by thiolase under physiological con-

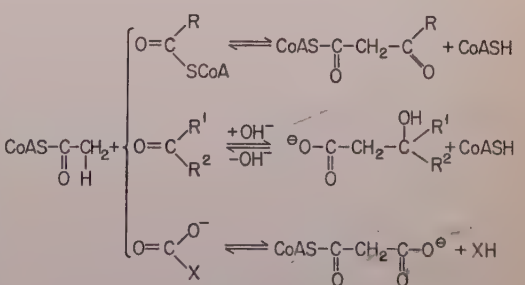


Fig. 10 Electrophilic substitution on the α-carbon of acyl—CoA.

ditions may be observed if the β-ketoacyl compounds produced by the reaction are removed by a subsequent reduction process (Lynen *et al.*, '52; Hele *et al.*, '57; Lachance *et al.*, '58). In this instance, the actual driving force for synthesis of the carbon chain is furnished by the two reduction steps necessary for the conversion of the keto acid to the saturated fatty acid (Lynen, '52–'53). Evidence for this was demonstrated in experiments performed by Seubert *et al.* ('57) in my laboratory with a particulate fraction from pig liver that catalyzed the hydrogenation of unsaturated acyl—CoA by TPNH (Langdon, '55). We coupled this system with purified thiolase, β-hydroxyacyl dehydrogenase, crotonase, and accessory enzymes for the regeneration of DPNH and TPNH (fig. 11) and found that labeled acetyl—CoA condensed with unlabeled capronyl—CoA, and that capryl, caprinyl, and higher acids appeared. There was, however, a flaw in these experiments, namely, the poor yield of naturally occurring longer chain fatty acids, e.g., stearic and palmitic (Seubert *et al.*, '57).

In this respect the mitochondrial syst that we studied differed from another zyme system that did incorporate acet into long chain fatty acids, which Bra and Gurin ('52) prepared in water-solu form by high-speed centrifugation of geon liver homogenates. For several ye the purification of the participating zymes of this system was studied in Gree laboratory ('56). Gibson *et al.* ('58) s ceeded in separating two enzyme fracti that together form almost exclusively p mitic acid from acetyl—CoA in the pr ence of ATP, TPNH, Mn^{++}, and bicarb ate. The stimulating effect of bicarbon on the synthesis of fatty acids in cell-f extracts was observed by Klein ('57) his experiments with yeast. Titchener *al.* ('58) found that the synthesis palmitic acid in the soluble system co be described by the following reacti 8 Acetyl—CoA + 16 ATP + 16 TPNH palmitate + 8 CoA + 16 ADP + 16 P + TPN. Bicarbonate does not enter i this equation, and in agreement with th experiments performed in the presence

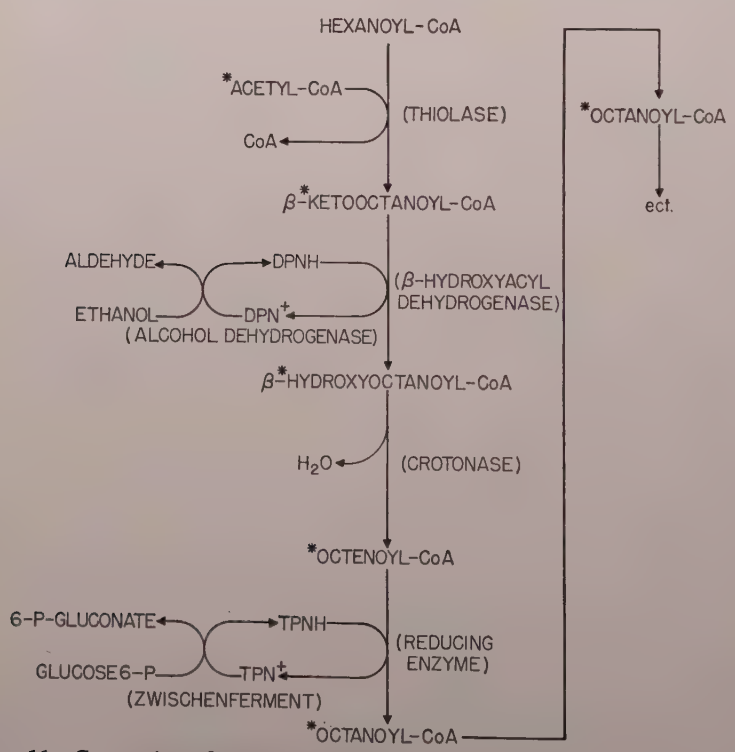


Fig. 11 Composite scheme for synthesis of fatty acids (reversible pathway).

O_2 do not result in the incorporation of ioactivity into palmitic acid (Gibson *et* '58). When Dr. Green presented these ılts at the last Gordon Conference on ide Metabolism in Meriden, N. H., I ıarked in the ensuing discussion that action of bicarbonate could be ex- ined by the intermediate formation of lonyl—CoA. This compound could be med from acetyl—CoA, bicarbonate, l ATP in analogy to the known carboxyl- ns of propionyl—CoA (Flavin *et al.*,) and β-methylcrotonyl—CoA (Lynen,). Two lines of reasoning led me to s idea. One arose strictly from the ther- dynamic considerations of C—C bond thesis. If malonyl—CoA were used for synthesis of β-keto acids instead of tyl—CoA, the thiolase equilibrium ght be shifted in favor of β-keto acid mation by the decarboxylation that ac- npanies the condensation (fig. 12). The

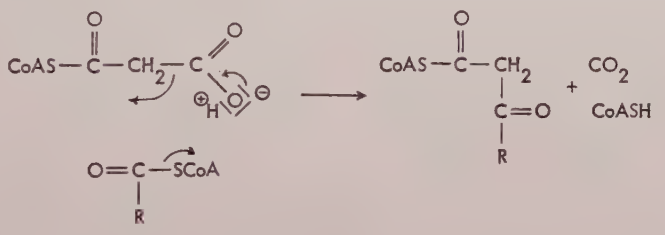


Figure 12

e-energy value of +6600 calories for thiolase reaction is an amount that reasonably be obtained from the de- boxylation. The condensation would be ilar to that mentioned in the formation δ-aminolevulinic acid, in which decar- ylation also occurs (see fig. 7). The ond reason for my suggestion was based the observation of Wakil *et al.* ('58) t one of the two enzyme fractions essary for the synthesis of palmitic acid tained biotin. It was known that the boxylation of propionyl—CoA or β- thylcrotonyl—CoA in animal tissues as l as in microorganisms depends on in supplementation (Lardy and Pean- y, '53; Lardy and Adler, '56). It seemed me that, for all biochemical reactions which biotin was found, "active CO_2" l hence carboxylation reactions were olved. In this connection, we found ing experiments on β-methylcrotonyl— CoA carboxylase from *Mycobacterium* spp. that this enzyme contains biotin as a prosthetic group. This will be discussed later.

My suggestion has proved to be correct. Brady ('58), who was present at the Gordon Conference, as well as Wakil ('58) in Green's laboratory have published brief communications on experiments in which malonyl—CoA was demonstrated as an intermediate substance in the conversion of acetyl—CoA to palmitic acid. These experiments were performed with liver and heart preparations. This finding has been verified in cell-free yeast extracts by studies in collaboration with my coworkers G. Domagk, I. Kessel, and M. Goldmann (unpublished data). Klein ('57) had demonstrated this source to be capable of incorporating acetate into long-chain fatty acids in the presence of ATP and bicarbonate. Domagk found it possible to sediment the active proteins of this system with ammonium sulfate at 55% saturation. With this crude enzyme preparation (AS_{0-55}), we could study the requirements for formation of palmitic acid; the results are shown in table 3. As in the studies with liver preparations (Gibson *et al.*, '58), ATP, Mn^{++}, CoA, and bicarbonate are required. TPN exhibits only a small although significant effect. The reason for this is that we routinely add DPN to the incubation mixtures. The Mn^{++} and bicarbonate requirements were studied carefully. Mg^{++} can largely substitute for Mn^{++}, but addition of Mn^{++} has an enhancing effect even in the presence of 0.01 *M* Mg^{++}. The activation effects of various concentrations of bicarbonate are shown in figure 13. We confirmed Klein's findings ('57) that, without bicarbonate, there is practically no fatty acid synthesis.

TABLE 3

The requirements for the formation of palmitic acid from acetic acid in the yeast system

Reactant omitted	Activity in long-chain fatty acids
	cts/min
—	14,700
Thiokinase	12,700
ATP	200
Mn^{++}	400
CoA	4,300
TPN and 6-phosphogluconate	10,000
HCO_3^-	4,000
AS_{0-55}	250

Protocol: 100 μmoles of K phosphate *p*H 7.5, 6 μmoles of 1-C^{14}-acetate (130,000 cts/min), 10 μmoles of ATP, 0.4 μmole of TPN, 0.4 μmole of DPN, 0.06 μmole of CoA, 0.5 μmole of $MnCl_2$, 0.6 μmole of 6-phosphogluconate, 75 μg of thiokinase, 260 μg of 6-phosphogluconate dehydrogenase, 9.4 mg of AS_{0-55}. Vol. 1.3 ml; 180 minutes at 30°C.

We next investigated the possibility of CO_2 fixation in this system by incubating mixtures containing unlabeled acetate and radioactive bicarbonate. Radioactivity curves of the acids isolated after hydrolysis of the CoA derivatives are shown on the upper part of figure 14. Below this are the results of a similar experiment per-

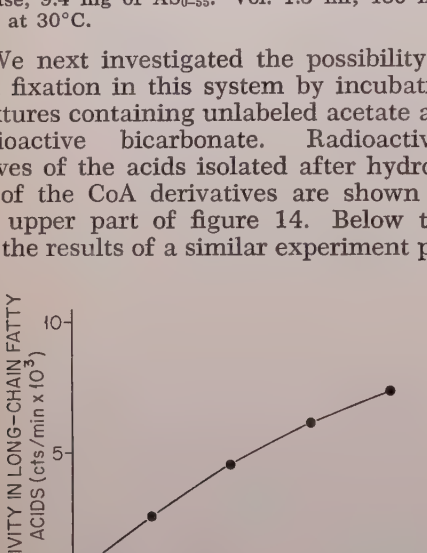


Fig. 13 Dependence of fatty acid synthesis on bicarbonate concentration. Incubation mixtures contained 100 μmoles of imidazole buffer (*p*H 7.1), 3 μmoles of 1-C^{14}-acetate (90,000 cts/min), 1 μmole of reduced glutathione, 5 μmoles of ATP, 0.2 μmole of TPN, 0.2 μmole of DPN, 0.02 μmole of CoA, 0.5 μmole of $MgCl_2$, 0.5 μmole of $MnCl_2$, 0.4 μmole of 6-phosphogluconate, 260 μg of 6-phosphogluconate dehydrogenase, 75 μg of acetic thiokinase, 4.6 mg of AS_{0-55}, and varying amounts of $KHCO_3$ as indicated in a total volume of 1.5 ml. After incubation for 60 minutes at 30°C., the fatty acids were isolated (Klein, '57) and counted.

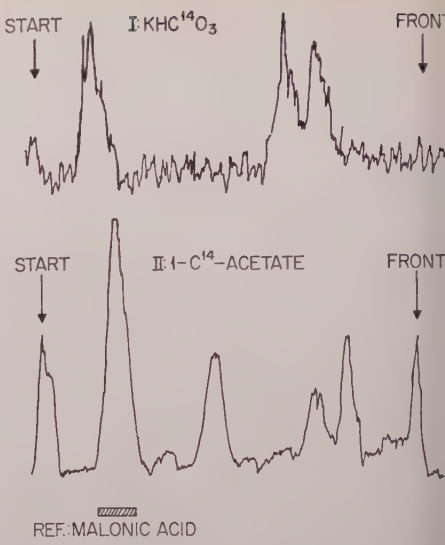
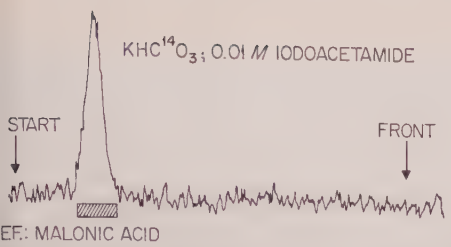


Fig. 14 Radioactive products formed fr labeled acetate and CO_2. Two tubes contain 200 μmoles of Tris (*p*H 7.1), 10 μmoles K_2Mg versenate, 6 μmoles of $MgCl_2$, 5 μmoles reduced glutathione, 15 μmoles of ATP, 2 μm of CoA, and 4 mg of enzyme (AS_{0-55}) in a t volume of 3.7 ml were incubated for 15 minu at 37°C. Additional contents of tube I: 4 μm of K acetate and 10 μmoles of $KHC^{14}O_3$ (1. 10^6 cts/min). Additional contents of tube 4 μmoles of K-1-C^{14} acetate (1.8×10^6 cts/m and 1.5 μmoles of $KHCO_3$. After alkaline drolysis, the mixtures were acidified and extrac with ether. The ether-soluble material chromatographed on S and S 2043 b paper w ethanol-NH_3-H_2O (20:1:4) and counted with recording strip counter.

formed with radioactive acetate and labeled bicarbonate. It can be seen th some radioactive peaks represent co pounds that contain radioactivity fr either bicarbonate or acetate. Since of these compounds was malonic acid, concluded that malonyl—CoA was form from bicarbonate and acetyl—CoA a was further metabolized by the yeast zymes. To prove this point, we studied inhibition of this system by iodoacetami We knew from previous experiments w β-methylcrotonyl—CoA carboxylase a with thiolase that the carboxylati by "active CO_2" are not inhibited this —SH poison (J. Knappe, E. Lor G. Jütting, and E. Ringelmann, unp lished results) but that the condensat reactions leading to the formation of keto acids are completely inhibited (Lyn



ig. 15 Effect of iodoacetamide on fatty acid thesis via malonyl—CoA. The reaction mix- containing 60 μmoles of Tris (*p*H 7.4), 10 oles of K_2Mg versenate, 10 μmoles of $MgCl_2$, moles of iodoacetamide, 6 μmoles of ATP, 0.2 ole of acetyl—CoA, 2.8 μmoles of $KHC^{14}O_3$ $\times 10^5$ cts/min), and 0.8 mg of enzyme $_{0-55}$) in a total volume of 1 ml was incubated 30 minutes at 37°C. The mixture was then ted and counted as in figure 14.

). As we had hoped, in the presence of 1 *M* iodoacetamide in this same yeast tem, the reaction of acetyl—CoA with arbonate and ATP was stopped at the el of malonyl—CoA. Only one radio- ive spot, that of malonic acid, was found the chromatogram of this experiment g. 15). By direct extraction of the un- lrolyzed enzyme mixture with phenol owed by paper electrophoresis, a com- nd was isolated having properties ntical to synthetic malonyl—CoA that Eggerer (unpublished results) in my oratory prepared. By this separation nique, variously labeled malonyl— A's were isolated. From 1-C^{14}-acetyl— A and unlabeled bicarbonate, we pre- ed carbonyl-labeled malonyl—CoA. m unlabeled acetyl—CoA and radio- ive bicarbonate, the carboxyl-labeled npound was obtained.

hese compounds have enabled us to ify a yeast fraction that catalyzes decarboxylation of malonyl—CoA to tyl—CoA and CO_2 in the ence of reduced pyridine leotide yet can be diverted to nthetic pathway by the addi- of DPNH or TPNH (F. Ly- and I. Kessel, unpublished ults). As shown in figure 16, equivalents of DPNH are dized per equivalent of mal- l—CoA. A similar rela- is found when TPNH is d. With the pyridine nucleo- -supplemented system, long- in fatty acids are synthe-

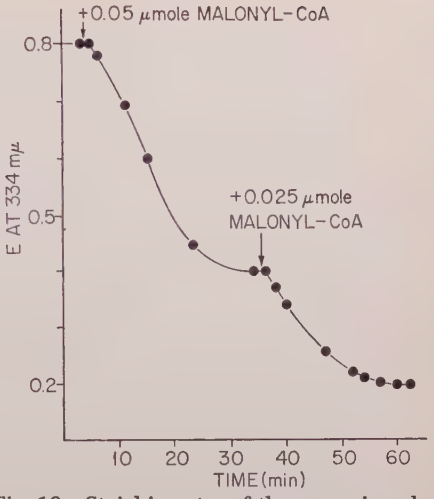


Fig. 16 Stoichiometry of the enzymic reduction of malonyl—CoA. A 1-cm cuvette containing 100 μmoles of K phosphate (*p*H 7.0), 10 μmoles of cysteine, 0.3 μmole of DPNH, and 0.7 mg of enzyme fraction in a total volume of 1.38 ml was incubated at 20°C. with additions as indicated. The enzyme was a preparation of AS_{0-55} that was further purified by dialysis, adsorption on Ca phosphate gel, and elution with 0.1 *M* Na pyrophosphate (*p*H 8.5), and refractionation with $(NH_4)_2SO_4$ (0–50%).

sized. These data lead us to the conclusion that ATP is required for only the carboxylation step. As with other ATP-mediated carboxylations, the products are probably ADP and phosphate. I might mention that palmitic acid synthesis from acetyl—CoA would then require 8 equivalents of ATP instead of the 16 reported by Titchener *et al.*, ('58) (see fig. 17). Since, after this activation, fatty acid synthesis proceeds without further need for ATP, we are left with the fascinating possibility that malonyl—CoA condenses with itself in a new

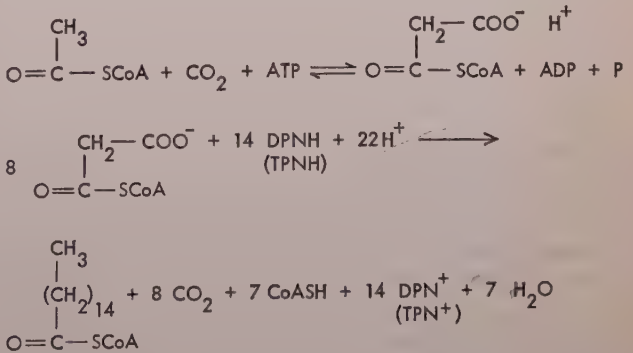


Figure 17

type of biological polymerization. Malonyl—CoA is an ideal starting monomer—energetically, it is "loaded" for formation of the C—C bond—kinetically, its decarboxylation assures the irreversibility necessary for a biosynthetic reaction.

What then is the nature of the polymeric product of malonyl—CoA? Experience with the biosynthesis of terpenes has taught that an insight into the nature of a repeating unit is often found in natural products. By examining a large number of polyterpenes, Ruzicka ('53) implicated an isoprenoid structure as the precursor. The important contributions of Collie ('07), Birch ('57), Robinson ('55), and Woodward ('56, '57) showed that another vast group of complex molecules occurring in nature could actually be considered as polyacetic acid derivatives. This group of substances includes a variety of phenols, pyrones, quinones, flavones, and other aromatic compounds as well as macrolides. Experimental verification exists in the demonstration of the incorporation of labeled acetate into some of these compounds (Birch, '56; Grisebach, '57). Just as isopentenyl pyrophosphate has proved to be the actual biological isoprenoid-condensing unit (see p. 33) we can now speculate that in analogy, malonyl—CoA may be the real condensing unit in the polyacetic acid series. Figure 18 indicates how malonyl—CoA might condense with decarboxylation to form a C_{16}-polyketomethylene acid and then be either reduced to palmitic acid or, in another biological system, cyclized to eleutherinol. If this scheme proves correct, we will have also the key for the biosynthesis of tetracycline and many other bacterial products (Woodward, '56, '57).

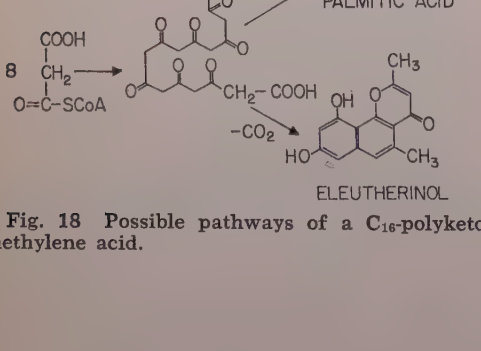


Fig. 18 Possible pathways of a C_{16}-polyketomethylene acid.

A variation of the polyacetic acid me
anism may exist in the as yet hypotheti
polymerization of the known carboxylat
product of propionyl—CoA, methylmalo
—CoA, to yield erythronolide, the aglyc
of erythromycin (fig. 19). Experimen
support for the existence of a p
pionate pattern in erythronolide (G
zon *et al.*, '56; Woodward, '56) w
supplied by experiments with labeled p
pionic acid (Vaněk *et al.*, '58). And s
may well be that the biosynthesis of ma
complex aromatic natural products is no
ing more than a modification of fatty a
synthesis, as Robinson ('55) and Wo
ward ('56) predicted.

With regard to the second category
electrophilic substitutions on the α-carb
of acyl mercaptans, the aldol-type c
densation (fig. 10), there is little of rec
interest. The failure to demonstrate
presence of the carbanion by deuteri
exchange in the presence of condens
enzyme (Marcus and Vennesland, '58)
been mentioned. Characteristically,
condensation is coupled with hydrolysis
the thioester bond of acetyl—CoA, wh
reacts as a carbanion. This hydroly
forces the reaction in the direction of s
thesis (Stern *et al.*, '52). The prototy
of these reactions, the synthesis of citr
from acetyl—CoA and oxaloacetate,
nevertheless be reversed to form acety
CoA from citrate if the concentrations
acetyl—CoA and oxaloacetate are kept
by coupling with other reactions. In t
manner, citrate may be used as ac
donor for aromatic amines (Stern *et*
'51) or for choline (Korkes *et al.*, '52).
the two other reactions, the synthesis
malate with glyoxylate (Ajl, '56; Ko
berg and Madsen, '58) and of HMG—C
with acetoacetyl—CoA (Rudney and
guson, '57; Lynen, Henning, *et al.*, '5
the reversibility cannot be demonstra
even in the presence of coupling syste
U. Henning (unpublished results) in
laboratory attempted to use HMG—C
as acetyl donor in the presence of HM
CoA-condensing enzyme, thiolase, and
lamine acetylase without success. T
brings us back to the mechanism of
drolysis of the thioester bond that is c
led to the condensation. The most li
possibility, that the reaction occurs in

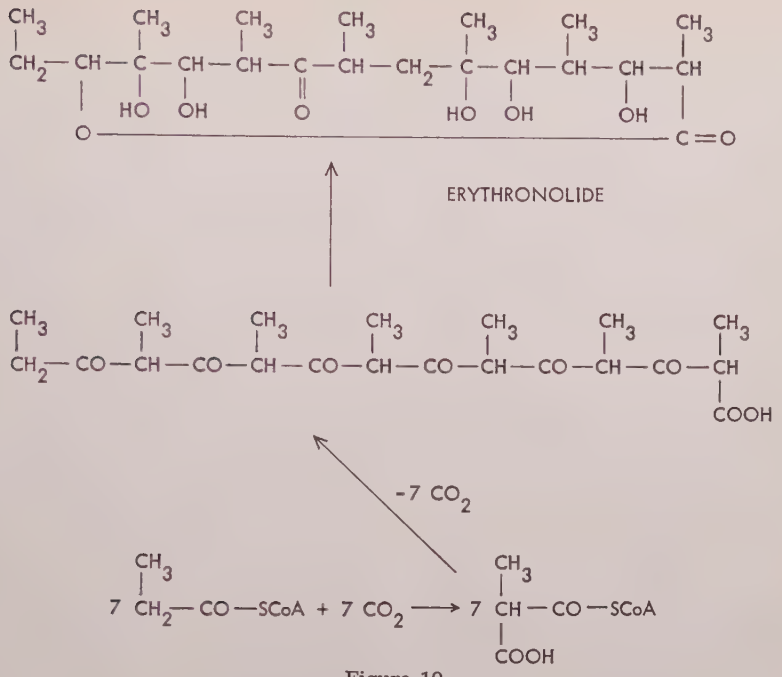


Figure 19

eps and that a CoA intermediate arises, cks experimental proof. Attention should drawn to the mechanism proposed by Eschenmoser and D. Arigoni (personal mmunication), in which the β-lactone an intermediate (fig. 20). This mechism would also explain the inability of etyl—CoA to pick up deuterium in the esence of condensing enzyme.

Whether the third type of electrophilic action of acyl—CoA that can make new -C bonds belongs in my discussion or one dealing with carboxylation and deboxylation is perhaps a moot point. I would like to discuss some aspects of these reactions since acyl—CoA is involved and neither CO_2 nor bicarbonate, but rather an "activated CO_2," participates here. Delwiche *et al.* ('54) found the first experimental evidence for formation of "active CO_2" in certain carboxylating and decarboxylating processes in studies on the decarboxylation of succinic to propionic acid in microorganisms. Shortly after this came the work of Bachhawat *et al.* ('54), and of Flavin *et al.* ('55), in which the participation of ATP in the carboxylation of β-hydroxyisovaleryl—CoA and propio-

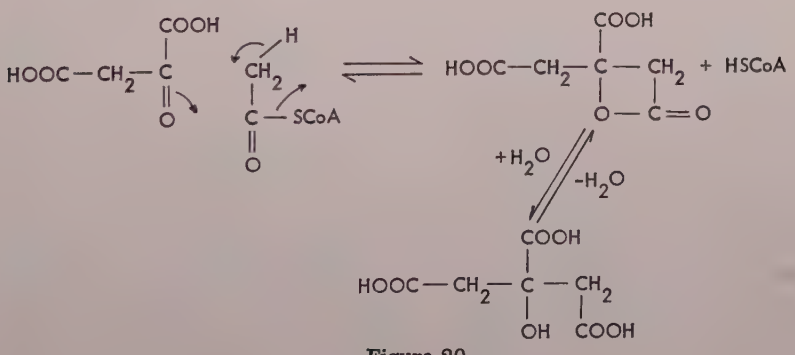


Figure 20

nyl—CoA was reported. Flavin *et al.* ('57) found in addition that the energy for the carboxylation of propionyl—CoA is furnished by the splitting of ATP into ADP and inorganic phosphate. Knappe ('57) demonstrated the same balance for carboxylation of β-hydroxyisovaleryl—CoA. In studies with an enzyme isolated from *Mycobacterium* spp., we found that the true CO_2 acceptor is methylcrotonyl—CoA and the product is β-methylglutaconyl—CoA (Knappe, '57; Lynen, '58b) (fig. 21).

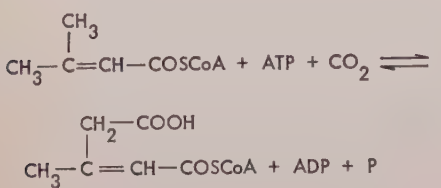


Figure 21

This revised scheme was also recently demonstrated in Coon's laboratory for the animal system (del Campillo-Campbell *et al.*, '59).

With these experiments, it becomes possible to view both of these reactions [and probably the conversion of acetyl—CoA to malonyl—CoA (see p. 41) as well] as occurring by a common mechanism: carboxylation on the α-carbon of acyl—CoA or on a "vinyl analog" α-carbon (Lynen, '58b) with the simultaneous splitting of ATP into ADP and inorganic phosphate. We have been studying the carboxylation of β-methylcrotonyl—CoA intensively. Mycobacteria grown on isovaleric acid served as a good enzyme source (Knappe, '57). After 150-fold purification of the enzyme (E. Lorch and G. Jütting, unpublished data), it was possible to demonstrate the reversibility of the reaction (table 4). Since β-methylglutaconyl—CoA is not available via synthetic routes, for these experiments HMG—CoA was converted to β-methylglutaconyl—CoA by addition of methyl glutaconase (Hilz *et al.*, '58).

HMG—CoA $\rightleftharpoons$ β-methylglutaconyl—CoA + H_2O

β-methylglutaconyl—CoA + ADP + P $\rightleftharpoons$ β-methylcrotonyl—CoA + CO_2 + ATP

ATP + glucose $\rightarrow$ ADP + glucose 6-phosphate.

The ATP produced was trapped by the hexokinase reaction and was assayed by means of glucose 6-phosphate dehyd genase and TPN (Kornberg, '50). So seems that among the biochemical re tions that lead to ATP, we can now add t decarboxylation of certain carboxylic ac (see also Tietz and Ochoa, '58). The che ical reaction by which decarboxylation p duces ATP or, conversely, by which t splitting of ATP facilitates carboxylati remained unknown. Flavin *et al.* ('5 and Bachhawat *et al.* ('56) pursued t idea that an "active CO_2" plays a ro Their suggestion, that a CO_2-phosphate, a CO_2-adenylate is involved seemed e tremely improbable from the chemic point of view.

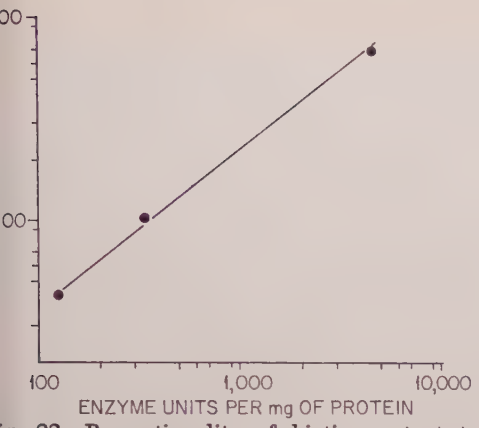
At this point, I would like to propose t working hypothesis that "active CO_2" is carbonic acid derivative of biotin, the su stance that was implicated in carboxy tion reactions by the pioneer work Lardy and Peanasky ('53), Lardy a Adler ('56), Chambers and Delwiche ('54 and Fischer ('55). Since it was know that biotin is bound by means of its c boxyl group to protein in biological m terial (György, '54), it was natural to su pose that β-methylcrotonyl—CoA carbo lase is a biotin enzyme and that biotin self is a functional prosthetic group. Th was in agreement with the observation Woessner *et al.* ('58) that the carboxyla is completely lacking in biotin deficien Biotin analysis of our purified carboxyla revealed that the biotin content increas as a function of purity of the enzyme (f 22). In the purest preparations made Lorch and Jütting (unpublished data) ratio of 1 mole of biotin per 344,000

TABLE 4

Formation of ATP by reversal of β-methylcroto —CoA carboxylation

Component omitted	Glucose 6-phosphate formed
	μmole
—	0.047
Carboxylase	0.008
Methylglutaconase	0.009
HMG—CoA	0.011

Protocol: 50 μmoles of K phosphate *p*H 10 μmoles of $MgSO_4$, 50 μmoles of glucose, 0 μmole of ADP, 0.1 μmole of HMG—CoA, 80 μ hexokinase, 50 μg of methylglutaconase, 25 μg β-methylcrotonyl—CoA carboxylase. Vol. 1.5 60 minutes at 37°C.



ig. 22 Proportionality of biotin content to ɣme purity. Biotin was measured by bioassay ɛhstein, '55). The enzyme was purified from extract of *Mycobacterium* grown on isovaleric . The first point on the graph represents surements on a fraction obtained after two $I_4)_2SO_4$ fractionations and a $MnCl_2$ step. The nd and third points represent measurements ractions obtained by further purification by ɔmatography on columns of DEAE-cellulose hydroxyapatite, respectively.

ɔrotein was found. The purified enzyme ld be inhibited by avidin and protected inst avidin by addition of free biotin. ıs we confirmed the concept that the in was actually related to enzyme ac- ɣy. As the result of further experi- ıts, we have derived the following re- ons which together comprise the car- ylation by ATP and biotin enzyme.

$$\xrightarrow{Mg^{++}}$$

+ biotin enzyme $\rightleftharpoons$ ADP ~ biotin enzyme + P

~ biotin enzyme + $CO_2 \rightleftharpoons$
CO_2 ~ biotin enzyme + ADP

~ biotin enzyme + β-methylcrotonyl—CoA $\rightleftharpoons$
biotin enzyme + β-methylglutaconyl—CoA

+ CO_2 + β-methylcrotonyl—CoA $\rightleftharpoons$
β-methylglutaconyl—CoA + ADP + P

individual reaction steps have been ıonstrated by exchange experiments nen and Knappe, '59). The specific bition by avidin gave valuable help. Ve were fortunate to find that free -biotin could act as a substrate for the ɔoxylase. The specificity of this reac- was demonstrated by the fact that -biotin and desthiobiotin were com- ely inactive. By experiments with $C^{14}O_2$ ırboxylated biotin derivative was de- ed and studied. On the basis of its nical properties, as well as theoretical nical considerations, we have derived a structure for this compound and therefore a model for the structure of "active CO_2." It is related to allophanic acid and has one of the configurations in figure 23.

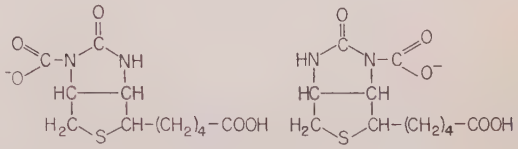


Figure 23

We cannot say which of the two nitrogen atoms is involved or whether ADP is bound to nitrogen or oxygen in ADP~biotin enzyme (fig. 24). In either case the interaction with CO_2 or bicarbonate could result in the formation of "active CO_2."

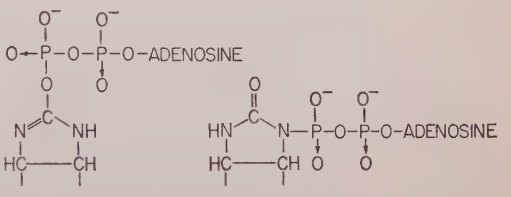


Figure 24

If we examine the special chemical properties of biotin that might make it suitable as an activator and carrier of CO_2, our attention is drawn to the acidic properties of urea nitrogen. The CO_2 adduct of biotin has then the properties of an acid anhydride. Thus its propensity for condensation to the α-carbon of acyl—CoA becomes understandable. The C—N bond is polarized in the direction of the nitrogen so that the CO_2-group can act as an acylating agent with the carbanion of acyl—CoA (fig. 25).

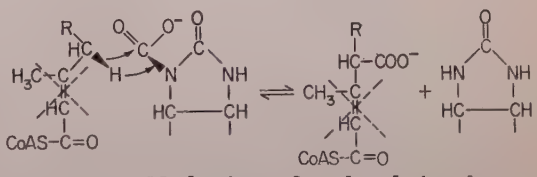


Fig. 25 Mechanism of carboxylation by "active CO_2."

This chemical mechanism holds not only for the carboxylation of β-methylcrotonyl—CoA, but also of propionyl—CoA and of acetyl—CoA. Wakil's demonstration ('58) of biotin in his acetyl—CoA carboxylase preparation and its inhibition by avidin confirms this.

It would seem then that from experimental as well as theoretical considerations, that we now have what is very likely the true structure of "active CO_2."

OPEN DISCUSSION

CALVIN[1]: What is the half life of the active CO_2 in water at neutral *p*H and room temperature?

LYNEN: It is very labile to acid. At *p*H 4.7 in 20 minutes, at 0°C. it is gone, but is quite stable to alkali at 0°, and for this reason we believe that it is linked to the nitrogen and not to the oxygen of the urea.

STROMINGER[2]: I missed how you prepared the CO_2 biotin.

LYNEN: We found that our carboxylase is able to substitute biotin for the substrate; so we can use free biotin as a carboxyl acceptor. The strange thing is that free biotin has some affinity for the enzyme and so it is carboxylated too. It is a poorer substrate than methylcrotonyl —CoA.

WOOD[3]: I congratulate you, Dr. Lynen, on this wonderful work. We have been interested in CO_2 fixation for many years, particularly in relation to the propionic acid fermentation. For a number of years it has been evident that a C_1 compound, which is not CO_2, is formed during the propionic acid fermentation (Wood and Leaver, '53). This becomes evident in the fermentation of pyruvate where, for every molecule of propionate that is formed, there should be one CO_2 fixed in oxaloacetate and again liberated in the decarboxylation of the succinate. If free CO_2 were involved there should be a turnover of CO_2. Experiments were done with unlabeled pyruvate and labeled bicarbonate, and the dilution of the C^{14}-bicarbonate was measured. It was found that a very small amount of $C^{12}O_2$ was liberated.

A related problem is the randomization of C^{14} in propionate. When a cell-free enzyme preparation from the propionic bacteria is incubated with β-labeled propionate, the C^{14} is rapidly randomized into the α position. Apparently a symmetrical C_4 dicarboxylic acid is formed; presumably the C_1 compound is present in catalytic amounts. By decarboxylation of the symmetrical C_4 there is randomization of the C^{14} in the propionate. There is little incorporation of CO_2 during this randomiz tion of C^{14}. Some of you may recall th we did mass spectrometer studies to pro that the randomization is not by a cycliz tion reaction and that there actually is C_1 cleavage during this randomizati (Pomerantz, '58).

More recently we have tested wheth this C_1, which apparently is formed fr succinyl—CoA, is transferred directly pyruvate to yield oxaloacetate. We fou with succinyl—CoA that is carboxyl label that β-labeled oxaloacetate is formed fr pyruvate. Apparently the C_1 that com off from the succinyl—CoA can be tra carboxylated to the pyruvate, yielding t β-labeled oxaloacetate and also carbox labeled propionate. I think these resu fit in very well with your experiments, L Lynen, and certainly fit in extremely w with the earlier experiments that Dr. C son and his coworkers have done. I shou say that Dr. R. W. Swick in our depa ment is doing this work and deserves mu credit.

LYNEN: We measured the ADP form tion during carboxylation by means of optical assay. The (+)-biotin is acti whereas (−)-biotin is not. Simple ethyle urea is also inactive. Homobiotin is son what active but much less than (+)-biot

[1] Melvin Calvin, University of California.

[2] Jack Strominger, Washington University, Louis.

[3] H. G. Wood, Western Reserve University.

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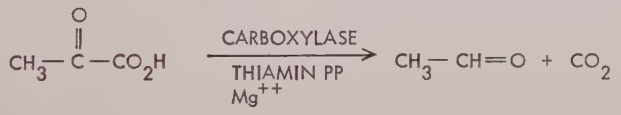
rboxylations and Decarboxylations[1]

MELVIN CALVIN AND NING G. PON
Department of Chemistry and Lawrence Radiation Laboratory, University of California, Berkeley

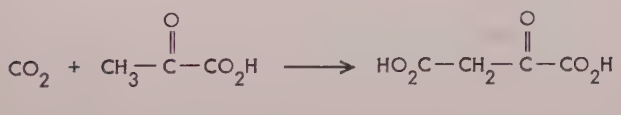
a symposium on the mechanism of me action, the nature of carboxylation decarboxylation reactions would natly be defined as those that are carried by enzymic means. We shall not to review all the carboxylation and rboxylation reactions that appear in literature but will omit from this dision the decarboxylation and carboxyn reactions involving the α-carboxyl p of amino acids. These decarboxylns are rather closely related to the samination reaction discussed by E. E. l in this Symposium—there are simicofactors involved—and so we will ude from our consideration the amino carboxylation and decarboxylation reons.

to form materials like carbamyl phosphates and urea groups. We will be concerned, then, primarily with the addition of CO_2 to some carbon skeleton, in which a new C—C bond is formed and in which a carboxyl group is created. Considerations of decarboxylation reactions will be in terms of what light they can throw on the nature of carboxylation reactions rather than for the sake of the decarboxylation reaction itself or for the sake of the completeness of the discussion.

In reviewing the mechanisms of such enzymic carboxylation reactions, we are struck by the fact that there seems to be no unequivocal description of a primary product formed between the enzyme, or a cofactor, and CO_2 prior to the appearance



(Reaction 4, table 1) (A)



(Reaction 5, table 1) (B)

Figure 1

urthermore, the interest in this subject, east in our laboratory, is primarily in s of carboxylation reactions rather decarboxylation reactions. We shall efore emphasize the reactions in which is added to another carbon atom to a new C—C bond, resulting in a carlic acid. This automatically will also ude from our discussion the carboxyl reactions in which CO_2 reacts with s other than carbon, such as nitrogen, of the new C—C linkage. For example, if we were to consider one of the longest known of carboxylation reactions (or decarboxylation reactions), namely, the decarboxylation of pyruvic acid by the enzyme carboxylase to give acetaldehyde and CO_2, we would find no indication in the literature of an intermediate (a carrier of CO_2) between pyruvic acid and the liber-

[1] The work described in this paper was sponsored by the U.S. Atomic Energy Commision.

ated CO_2 (Green *et al.*, '41) (see fig. 1A). A reaction of the reverse type that has been studied—the carboxylation of pyruvic acid to oxaloacetic acid—leads to the formation of a new C—C bond (Kaltenbach and Kalnitsky, '51) (see fig. 1B). Again, in this reaction there is no evidence of an intermediate involving CO_2 between the CO_2 and the new C—C bond that is formed in the oxaloacetic acid (OAA). That is, the CO_2 may or may not be bound to enzymes or cofactors before it is attached to the pyruvate skeleton, if indeed it is, we have no description of such an intermediate.

This is curious because, in most other enzymic reactions in which a small group is picked up and handed on to be combined with a larger one, or with another one, in general, intermediates have appeared in which the group to be transferred is bound to the enzyme or to a catalytic amount of cofactor that, in conjunction with the enzyme, moves the group around before it appears in its subsequent substrate form. Therefore, to obtain a clue as to how such enzymic carboxylation and decarboxylation reactions might occur, we have to surmise from the mechanisms proposed for purely chemical systems.

the organometallic compound. This wo for a number of other organometallic terials as well (fig. 2). The other type

Figure 2

purely chemical carboxylation react with which we are familiar is of quite a ferent sort; it involves the carboxylation the metal salt of an enol or phenol, par ularly the latter (Pedersen, '47; Brov '51). For example, sodium phenol treated with CO_2 at elevated temperatu and pressures will produce sodium sali ate (fig. 3).

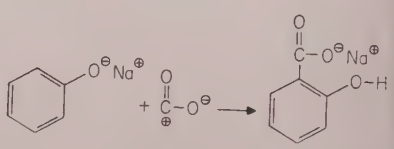


Figure 3

A relative of this enolate carboxylat may very well be found in the carboxy tion of metal salts of nitroalkanes to fo the metal chelate of the carboxylic a (Stiles and Finkbeiner, '59) (fig. 4).

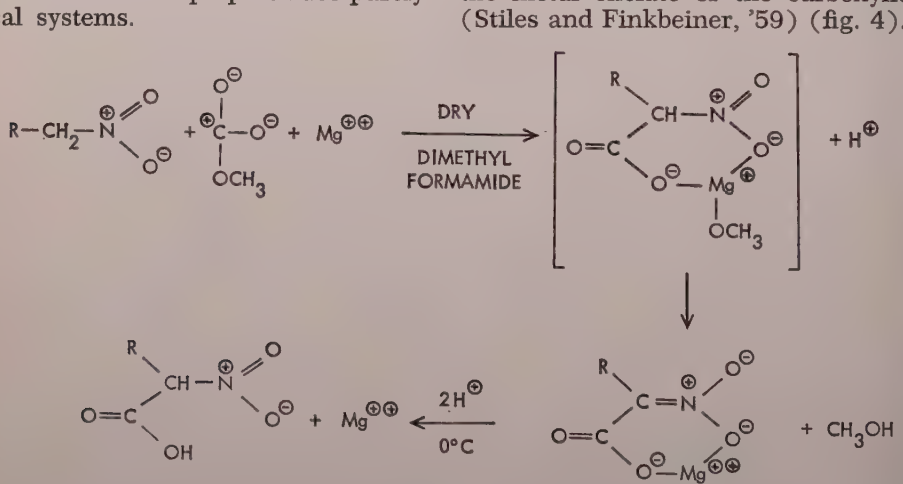


Figure 4

Two purely chemical carboxylations are well known. One is the reaction of an organometallic compound, such as a Grignard reagent, with CO_2 to form a new C—C bond in which the C=O grouping of the CO_2 is presumably inserted between the carbon (carbanion) and the metal of

The study of decarboxylation reactio has given a clue to the possible gene character of the nature of the carboxy tion reaction itself. This has been und taken primarily in connection with very easy decarboxylation of β-keto aci which, when heated, readily lose a mo

of CO_2 to give the corresponding ke- (fig. 5). The R group may be a hy-

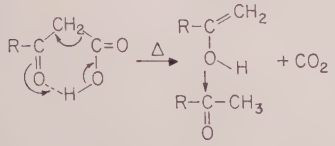


Figure 5

yl, as in malonic acid, a methyl group, n acetoacetic acid, or a carboxyl, as in , and so on. The decarboxylation reac- will, in general, lead ultimately to the esponding carbonyl compound. A good of work has been done on the mech- m of the decarboxylation, particularly cetoacetic and oxaloacetic acids and derivatives (fig. 6). β-Keto acids are

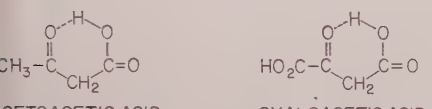


Figure 6

ent in both cases. The literature in- tes that, in some cases, the primary uct is an enol of the corresponding ne, which then undergoes tautomeri- n. In some cases, the decarboxylation s to go as fast, or faster, when e is no enolizable hydrogen in the al keto acid; e.g., the experiments of rsen ('34) and Westheimer and s ('41). This has led to the opinion the primary product is at least a ogen-bonded carbonyl rather than a enol. The truth of the matter may well lie in a compromise between the points of view. On some occasions the ary product of the decarboxylation presumably be closer to the enol, and thers (sterically hindered) the pri- product may very well be simply a ogen-bonded ketone. If an enol or c type of compound is the primary ue for the breaking of the C—C in carboxylation reaction, we can then d this notion to the reverse operation. , where CO_2 is added to make a new C bond, the first requirement would to be a system that contains a true, least a potential, enolic system, pre- ory to the addition of the CO_2 to it. is simply a statement that we may expect the same transition state to participate in the reaction in either direction (fig. 7).

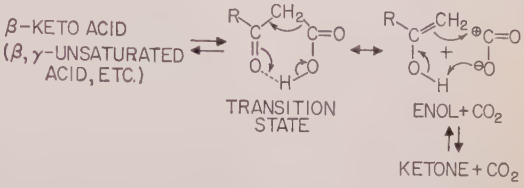


Figure 7

TYPES OF CARBOXYLATION (DECARBOXYLATION) REACTIONS

An examination of those enzymic reactions for which the requirements are fairly well established (table 1) leads to the classification of the carboxylation, or reversible decarboxylation, reactions associated with them into three general types. Let us formulate them in terms of carboxylation reactions rather than decarboxylation reactions, even though in some cases they are known and have been studied primarily as decarboxylation reactions. We have tried to organize the best known of the carboxylations in these terms: those having an energy requirement in the form of ATP clearly established; those having a requirement for a reduced pyridine nucleotide; and those having no apparent extra energy requirement.

Carboxylation reactions requiring adenosine triphosphate as energy source

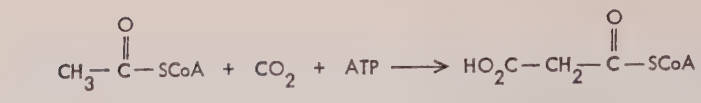
ATP-requiring carboxylations are as follows: the carboxylation of acetyl thiol ester in the form of acetyl—CoA, to yield malonyl—CoA (Wakil, '58; Formica and Brady, '59) (fig. 8A). Corresponding to this in form would be the carboxylation of the next higher fatty acid ester, propionyl —CoA, to give methylmalonyl—CoA (Flavin *et al.*, '57; Tietz and Ochoa, '58). This, again, is an α carboxylation (fig. 8B). Another case for which the purely formal reaction has been well established but for which the mechanism is still a subject of some discussion is the apparent direct carboxylation, not of α-carbon atoms but of a γ-carbon atom of a thiol ester in the form of β-methyl-β-hydroxybutyryl—CoA (Bachhawat *et al.*, '56; Bachhawat and Coon, '57, '58) (fig. 9A).

TABLE 1

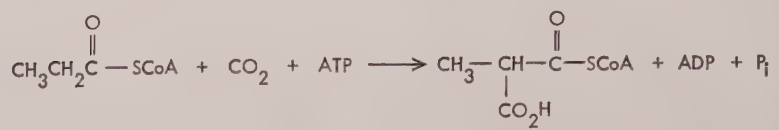
Carboxylation (decarboxylation) reactions

Reaction	References	Enzyme	Substrate	Product	Metabolic pathway	Cofactors or additions
1	Jagannathan and Schweet, '52 Korkes *et al.*, '51 Gunsalus, '54	Pyruvic dehydrogenase (pyruvic oxidase)	Pyruvic acid	Acetyl—CoA	Glycolysis to TCA	DPN^+, CoA, thioctic acid, TPP, Mg^{++}
2	Sanadi *et al.*, '52 Burton and Krebs, '53 Kaufman *et al.*, '53 Gunsalus, '54	α-Ketoglutaric dehydrogenase (α-ketoglutaric acid oxidase)	α-Ketoglutaric acid	Succinyl—CoA	TCA	DPN^+, CoA, TPP, thioctic acid, Mg^{++}
3a	Meister, '57	α-Keto acyl dehydrogenase	α-Keto-β-methylvaleric acid	α-Methylbutyryl—CoA	Isoleucine metabolism	CoA, DPN^+, thioctic acid, TPP
3b	Meister, '57	α-Keto acyl dehydrogenase	α-Ketoisocaproic acid	Isovaleryl—CoA	Leucine metabolism	CoA, DPN^+, thioctic acid, TPP
3c	Meister, '57	α-Keto acyl dehydrogenase	α-Ketoisovaleric acid	Isobutyryl—CoA	Valine metabolism	CoA, DPN^+, thioctic acid, TPP
4	Green, '41	Pyruvic carboxylase	Pyruvic acid	Acetaldehyde	Glycolysis	TPP, Mg^{++}
5	Kaltenbach and Kalnitsky, '51 Vennesland *et al.*, '49	Oxaloacetic decarboxylase (oxaloacetic carboxylase)	Oxaloacetic acid	Pyruvic acid	TCA to glycolysis	Mn^{++}
6	Neuberger *et al.*, '56	α-Amino-β-ketoadipic decarboxylase	α-Amino-β-ketoadipic acid	δ-Aminolevulinic acid	Porphyrin metabolism	
7	Ochoa and Weisz-Tabori, '58 Siebert *et al.*, '57	Isocitric dehydrogenase	D-Isocitric acid	α-Ketoglutaric acid (α-KG)	TCA	TPN, Mn^{++}
8	Saz and Hubbard, '57 Ochoa *et al.*, '48	Malic enzyme (TPN-malic dehydrogenase)	Malic acid	Pyruvate	TCA to glycolysis	TPN^+ (plants and animals), DPN^+ (bacteria), Mn^{++}
9	Axelrod *et al.*, '53 Cohen, '54 Horecker and Smyrniotis, '52	Phosphogluconic dehydrogenase	6-Phosphogluconic acid	Ribulose 5-phosphate	HMP (Shunt)	Mg^{++} or Mn^{++}, TPN^+
10	Davies, '43	Acetoacetic acid decarboxylase	Acetoacetic acid	Acetone	Fatty acid metabolism	
11	Zelitch and Ochoa, '53 Kun *et al.*, '54	Glycolic acid oxidase	Glycolic acid	Formic acid	———	
12a	Mehler, '56 Hankes and Henderson, '57	Picolinic carboxylase	3-Hydroxyanthranilic acid	Picolinic acid	Tryptophan metabolism	Fe^{++}, O_2 ?

14	Bachhawat and Coon, '58 Woessner *et al.*, '58 Bachhawat and Coon, '57 Bachhawat *et al.*, '54 Bachhawat, Woessner, and Coon, '56 Bachhawat, Robinson, and Coon, '56	Hydroxyisovaleryl—CoA	β-Hydroxyisovaleryl—CoA	β-Hydroxy-β-methylglutaryl—CoA	Leucine metabolism	ATP, HCO_3^-, Zn^{++}
15	Flavin *et al.*, '56, '57 Tietz and Ochoa, '58	Propionyl—CoA carboxylase	Propionyl—CoA	Succinyl—CoA	Fatty acid metabolism	ATP, HCO_3^-, Mg^{++}
16	Wakil, '58 Formica and Brady, '59		Acetyl—CoA	Malonyl—CoA	Fatty acid metabolism	Mn^{++}, ATP, HCO_3^-
17	Jones *et al.*, '55 Grisolia and Cohen '53 Reichard, '57		Ammonia	Carbamyl phosphate	Urea cycle	Mg^{++}, ATP, acetylglutamic acid
18	Kurahashi *et al.*, '57 Tchen and Vennesland, '55	Phosphoenolpyruvic carboxylase	Phosphoenolpyruvic acid	Oxaloacetic acid	Glycolysis to TCA	IDP (animal), ADP (plant), Mn^{++}
19	Tchen *et al.*, '55 Bandurski and Greiner, '53	Phosphoenolpyruvic carboxylase	Phosphoenolpyruvic acid	Oxaloacetic acid	Glycolysis to TCA	None
20	Lukens and Buchanan, '57		5-Aminoimidazole ribonucleotide	5-Amino-4-carboxyimidazole ribonucleotide	Purine synthesis	
21	Weissbach *et al.*, '56 Quayle *et al.*, '54 Mayaudon *et al.*, '57 Mayaudon, '57 Hurwitz *et al.*, '56	Carboxydismutase	Ribulose 1,5-diphosphate	3-Phosphoglyceric acid	Photosynthesis	Mg^{++}
22	Knappe and Lynen, '58 Lynen *et al.*, '59		β-Methylcrotonyl—CoA	β-Methylglutaconyl—CoA	Leucine metabolism	

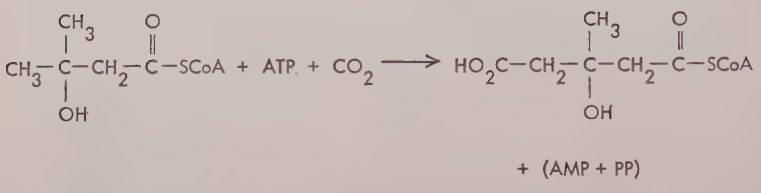


(Reaction 16, table 1) (A)

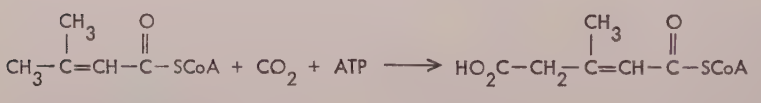


(Reaction 15, table 1) (B)

Figure 8



(Reaction 14, table 1) (A)



(Reaction 22, table 1) (B)

Figure 9

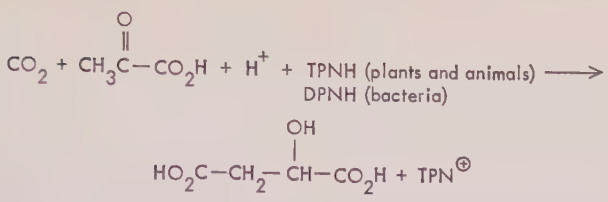
A reaction that seemed to be closely related to the one just described and believed to be part of the same system is the carboxylation of β-methylcrotonyl—CoA (Knappe and Lynen, '58; Lynen *et al.*, '59) (fig. 9B). This then would again lead to a carboxylation product in which the carboxyl group appeared on the carbon atom γ to the carboxyl group itself.

One other reaction requiring ATP (which we have agreed to pass over) is the formation of carbamyl phosphate, which does not involve the formation of a new C—C bond and therefore falls outside the field of this discussion. These, then, are all the reactions in which the formal creation of the C—C bond via carboxylation is known to require the presence of adenosine triphosphate.

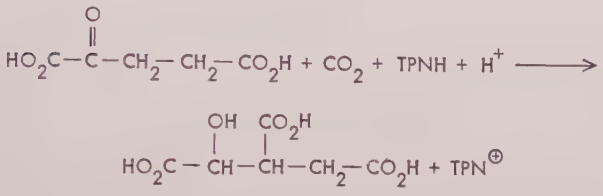
Carboxylation reactions requiring redu pyridine nucleotide as energy source

A second type of carboxylation reac requires TPNH but not ATP. These r tions are, in general, carboxylations β carbonyl group and do not lead to th keto acid but rather to the β-hydroxy a There are at least three such clear cases. The first is the carboxylation pyruvate itself with TPNH to give, in case, malic acid directly (Ochoa *et* '48; Saz and Hubbard, '57) (fig. 1 The enzyme that forms the malic does not make free OAA as a precu to the malic acid.

An analogous TPNH-dependent reac is the carboxylation of ketoglutaric to produce a β-hydroxy acid (Ochoa

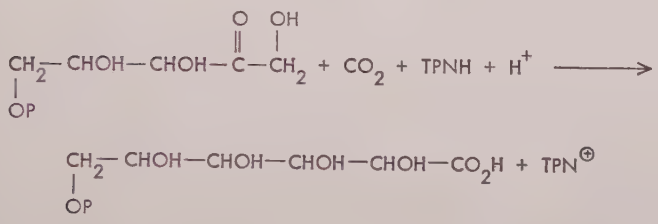


(Reaction 8, table 1) (A)



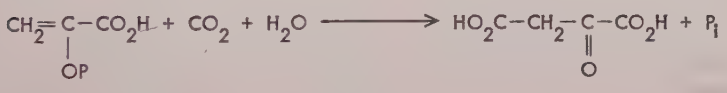
(Reaction 7, table 1) (B)

Figure 10



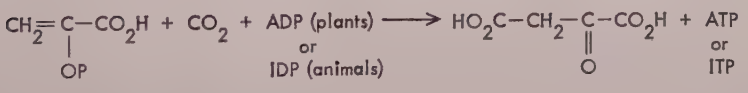
(Reaction 9, table 1) (A)

Type (1)



(Reaction 19, table 1) (B)

Type (2)



(Reaction 18, table 1) (C)

Figure 11

sz-Tabori, '48; Siebert *et al.*, '57) (fig.
).

third reaction that may belong to
class is the reductive carboxylation
ibulose 5-phosphate to 6-phosphoglu-
c acid (Horecker and Smyrniotis, '52; Cohen, '54) (fig. 11A). It is not certain whether an intermediate, 3-keto-6-phosphogluconate free of the enzyme, is involved. In any case, the reduction is necessary to show the reaction as a carboxylation.

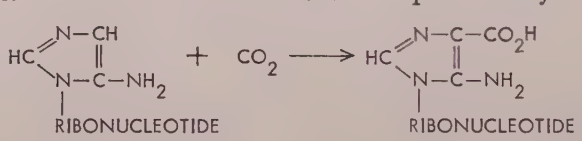
These three reactions all require additional sources of energy in the form of reduced pyridine nucleotide.

Carboxylation reactions that have no apparent extra energy requirement

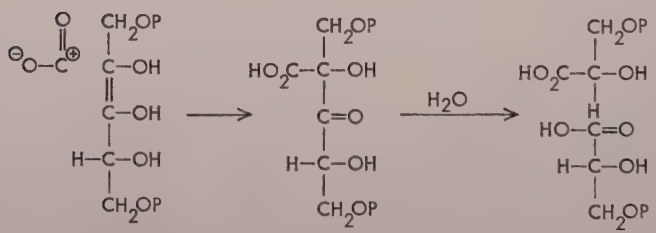
There are three reactions in which a new C—C bond is known to form without addition or direct participation, so far as we can tell, of ATP or reduced pyridine nucleotide. The first is the carboxylation of phosphoenolpyruvate. This reaction can, in turn, be subdivided into two parts —two different kinds of carboxylation: (1) one in which OAA and P_i are produced directly (Bandurski and Greiner, '53; Tchen *et al.*, '55) and (2) one in which the phosphate, instead of appearing as P_i directly, is picked up by ADP to form not only OAA but also ATP as the other product (Tchen and Vennesland, '55) (fig. 11B, C). These are two different carboxylation reactions requiring two different carboxylation enzyme systems.

phoglyceric acid) is the carboxylation ribulose diphosphate (Hurwitz *et al.*, '5 Weissbach *et al.*, '56; Mayaudon *et al.*, '5 (fig. 12B).

We therefore have at least three kin of carboxylation reactions, and each quires a source of energy to produce new C—C bond. The source of energy the case of the ATP requirement is viously ATP itself and in the case of pyridine nucleotide requirement is the duced pyridine nucleotide. In the th type of carboxylation reaction that quires neither ATP nor reduced pyridi nucleotide, the source of the energy is substrate itself. The substrate itself is ready in an "active" form in the se that it is unstable with respect to the m stable isomers. For example, in the ph phoenolpyruvate, the energy is stored the form of enol phosphate; in the imi zole, there is the carboxylation of an e amine; and in the ribulose diphospha there is presumably the carboxylation



(Reaction 20, table 1) (A)



(Reaction 21, table 1) **Figure 12** (B)

The second reaction that requires no ATP is the one in which we directly carboxylate an aminoimidazole ribonucleotide derivative of this character (Lukens and Buchanan, '57) (fig. 12A). The CO_2 adds at the C-4 to give the 4-carboxy-5-aminoimidazole ribonucleotide.

The third major carboxylation that does not require ATP or reduced pyridine nucleotide (at least *in vitro* to produce phos-

the noncyclic form of the ribulose, wh is constrained to go through an ene-d since cyclic acetal formation is prohibi by small ring size.

CARBOXYLATION REACTION MECHANISMS

Enol carboxylations

We would like to bring into harmony least two of these classes; we may not

le to bring the third one into harmony th the other two. Let us see if we can rmulate a reaction mechanism that uld be common at least to two types, the ol carboxylation and the reduced pyri- ne nucleotide-requiring one. No further scription is required for the carboxyla- n of the enol forms since they already present the model types that we spoke as being the kind of primary product of carboxylation in ordinary chemical de- rboxylation and also of the two cases chemical carboxylation with which we e familiar.

Thus we can describe the carboxylation phosphoenolpyruvate, according to Ven- sland and coworkers (Tchen *et al.*, '55) a direct carboxylation of the enol, lead- g to ejection of P_i and the direct forma- n of OAA (see fig. 11B). The analog this, one in which some acceptor other an water is required for the P_i (e.g., P or ADP), might be considered as a ore highly evolved system in which some the energy stored in the enol phosphate conserved in the ATP or ITP, as the se may be, for further use. In the case the ribulose, there is a direct carboxyl- ion of an enol form, which remains rgely in the enol form because of its in- ility to form the furanoside ring, there ing only three carbon atoms free and ailable for such ring formation. The minoimidazole carboxylation would cor- spondingly lead to the ketimine, which uld tautomerize because of the cyclic njugated structure to give the carboxyl- ed aminoimidazole (fig. 13).

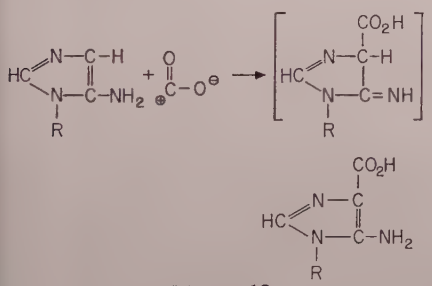


Figure 13

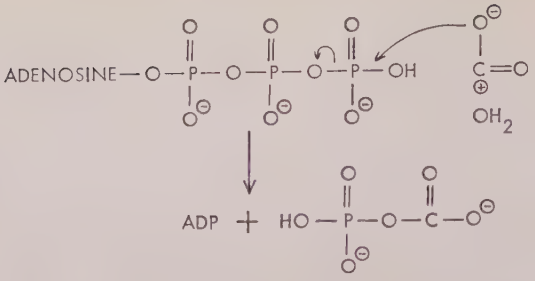
Reduced pyridine nucleotide-dependent reactions

The second type of carboxylation reac- on that should be included in this classi- fication is the one requiring reduced pyri- dine nucleotide as its energy source. For example, free pyruvic acid can be car- boxylated to form malic acid directly when TPNH is present (Ochoa *et al.*, '48; Saz and Hubbard, '57). Presumably, this will also go by way of the enol form on the enzyme, liberated only as the free malic acid after reduction by the TPNH required for this enzyme system. A similar mech- anism could be suggested for the formation of isocitric acid from ketoglutaric acid, again with TPNH as the energy source needed to complete the carboxylation re- action.

Adenosine triphosphate-dependent carboxylations

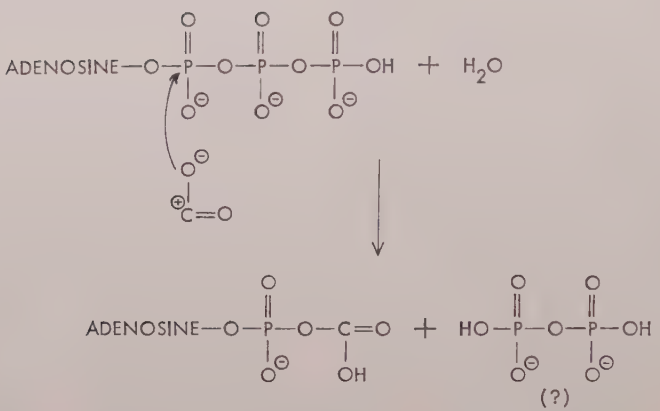
Let us now examine the class of car- boxylation reactions that require ATP to see how this energy supplied by the ATP actually performs its function in produc- ing the carboxylation reaction. Two sug- gestions have been made with regard to this function. The first concerns the car- boxylation of propionyl—CoA to form methylmalonyl—CoA (Flavin *et al.*, '57) and involves the primary activation of CO_2 to form phosphoryl carbonate. This phosphoryl carbonate would then be the "active" CO_2 that would carboxylate the α position of the propionyl—CoA to form methylmalonyl—CoA (fig. 14; phosphoryl carbonate anion). A separate enzyme is presumed to "activate" the CO_2 to form the phosphoryl carbonate. Another enzyme for the carboxylation itself (of propionyl— CoA) and a third for the isomerization of the product formed, methylmalonyl—CoA to succinyl—CoA, have also been separated (Flavin *et al.*, '57; Beck *et al.*, '57).

The second possibility for the function of ATP in a carboxylation reaction con- cerns the carboxylation of hydroxyisoval- eryl—CoA to form the β-methyl-β-hydroxy- glutaryl—CoA (Woessner *et al.*, '58). Woessner *et al.* suggested that the ATP reacts with the carbonate to form adenyl carbonate and pyrophosphate rather than splitting the other way, as in the first suggestion. Then the adenyl carbonate, called the "active" CO_2, apparently car- boxylates the hydroxyisovaleryl—CoA to



PHOSPHORYL CARBONATE ANION

Figure 14



ADENYL CARBONATE

Figure 15

form the hydroxymethylglutaryl—CoA (fig. 15).

In the other two carboxylation reactions, namely, that of acetyl—CoA to form malonyl—CoA (Wakil, '58; Formica and Brady, '59) and that of β-methylcrotonyl—CoA to form glutaconyl—CoA (Knappe and Lynen, '58), no direct evidence is available concerning the nature of the ATP requirement.[2]

It is perhaps worth pointing out at this juncture that neither of these two "active" CO_2 products has been isolated or demonstrated directly in the enzyme preparations. Coon and associates (Bachhawat, Woessner, and Coon, '56; Bachhawat and Coon, '57) synthesized the adenyl carbonate ethyl ester, using the silver salt of adenylic acid and ethylchlorocarbonate. The crude product of that reaction was claimed to substitute for the ATP requirement in the carboxylation of the hydroxyisovaleryl—CoA. This statement was made in a brief communication and no amplification has yet appeared, so perhaps we had better reserve judgment for the moment and see if some other unifying mechanism may be devised to account for the ATP requirement that would bring the ATP-requiring carboxylation reactions into a coherent pattern with the other two groups that we have already described as enol carboxylations.

Unifying mechanism

A rather obvious mode of action suggests itself, which already has its analog in the formation of phosphoenolpyruvic acid by pyruvic kinase (Lardy and Ziegler '45) as a prelimnary step to the carboxylation of phosphoenolpyruvate (Tietz and Ochoa, '58). This is the formation of the

[2] Paragraph 3 page 63 and continuing to paragraph 4 page 64 were added subsequent to presentation of the paper by F. Lynen (this Symposium).

ıol phosphate of the thiol esters men- ɔned as those requiring ATP for their ırboxylation. Formation of the enol phos- ıate of the thiol esters, acetylthiol—CoA ıd propionyl—CoA, as well as that of β- ethylcrotonyl—CoA, seems to be per- ctly straightforward. The β-methylcro- nyl—CoA would be a "vinylogous" enoli- ıtion on the γ methyl group, leading di- ctly to a "vinylogous" enol that would subject to carboxylation in the usual ay (fig. 16).

The case of the β-hydroxyisovaleryl—CoA, however, requires some further discussion. If, as is suggested by the work of Coon and coworkers ('56) and Lynen (Knappe and Lynen, '58), these are two independent enzyme systems and if we accept Lynen's evidence that his system, beginning with hydroxyisovaleryl—CoA, involves at least four stages, namely, (1) dehydration to β-methylcrotonyl—CoA, (2) the activation step, (3) the carboxylation step to β-methylglutaconyl—CoA, and (4)

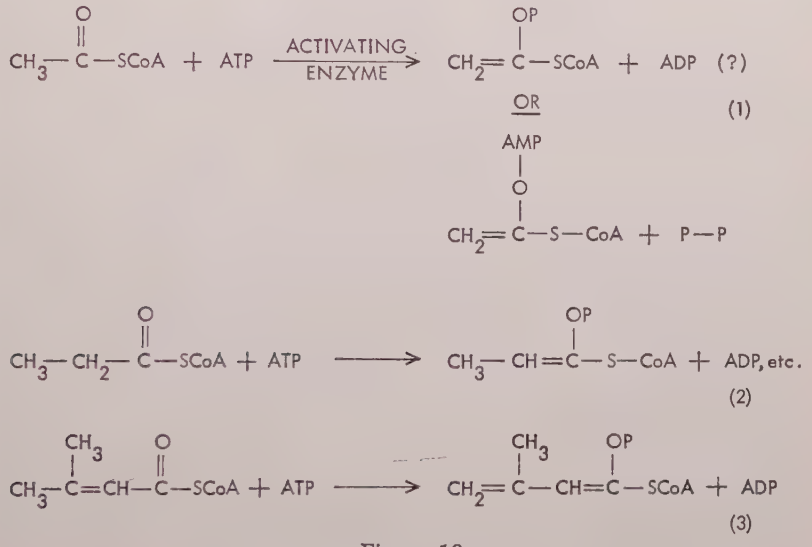


Figure 16

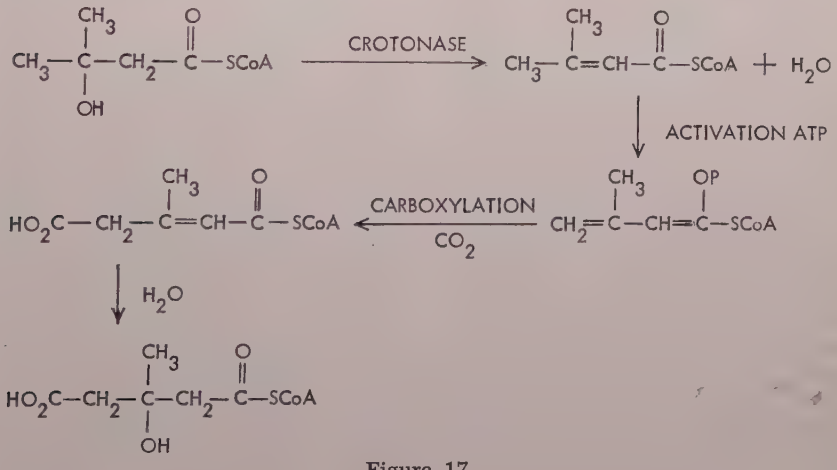


Figure 17

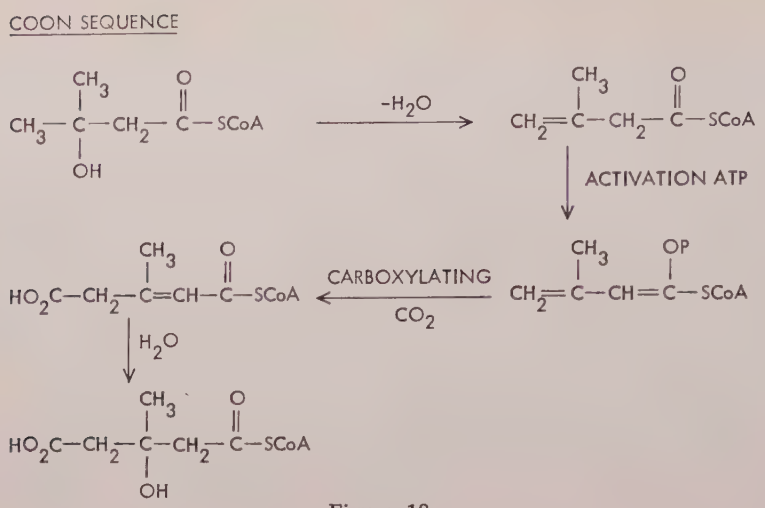


Figure 18

a rehydration to give β-hydroxy-β-methylglutaryl—CoA, then a somewhat different route must be devised for Coon's enzyme, which presumably functions in the absence of crotonase, the hydration-dehydration enzyme relating isovaleryl—CoA and β-methylcrotonyl—CoA (fig. 17). The most obvious suggestion would be that the Coon system involves a dehydration of the hydroxyisovaleryl—CoA in the opposite, or nonconjugated, way to give β-methylvinyl acetic acid rather than the crotonic acid (fig. 18). This would then be followed by activation to produce the conjugated dieneol, the same conjugated dienol as would be obtained from the crotonic acid. This process would then undergo the carboxylation and hydration as before, thus bringing both systems into the same form of carboxylation reaction.

Hence we have brought the three types of carboxylation reactions discussed into the same form, namely, that of the attack upon an enol by CO_2 (or bicarbonate ion) in its carbonium ion manifestation, leading directly to the formation of a C—C bond. An exactly similar formulation may be achieved for amino acid decarboxylation in which the oxygen atom of the enol is replaced by a nitrogen atom (Mandeles *et al.*, '54).

Two peripheral observations in connection with the proposed "active" CO_2 remain to be accounted for: first, the CO_2-dependent formation of phosphoryl fluori from ATP and fluoride ion under the i fluence of the fluorokinase (pyruvic ki ase) enzyme (Tietz and Ochoa, '58) wi the formation of ADP as the other produ (fig. 19A). The second is the appare hydrolysis of the ATP by the "CO_2-activ ing" enzyme of Coon (Bachhawat a Coon, '58), which requires hydroxylami and CO_2, leading to AMP and some py phosphate-like material, perhaps ph phoryl hydroxylamine. These reactions a presumed to take place in two stages (f 19B).

In both these cases, the fluoride and t hydroxylamine, respectively, seem to substitute acceptors of the "active" C in place of the natural acceptor, name the thiol esters. Also, in each case, t first reaction must be reversible and, this is so, we would indeed expect a ra exchange of carbon-labeled ADP with AT at least in the fluorokinase reaction. T has not been observed (Tietz and Och '58). It should also be pointed out th separation of the propionyl—CoA carbox ation system from purified fluorokin (Tietz and Ochoa, '58) seems possib

An alternative explanation for th two CO_2-dependent side reactions wo be as follows: The activating enzymes, kinases, in both cases would be conceiv of as ATP-activating enzymes in which ATP is prepared for its reaction with

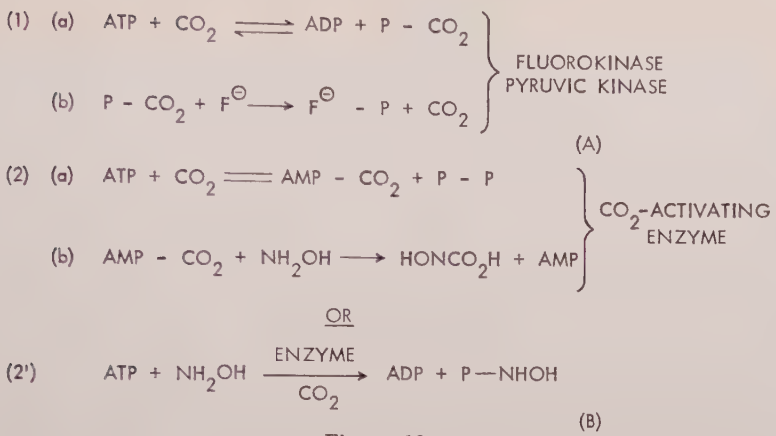


Figure 19

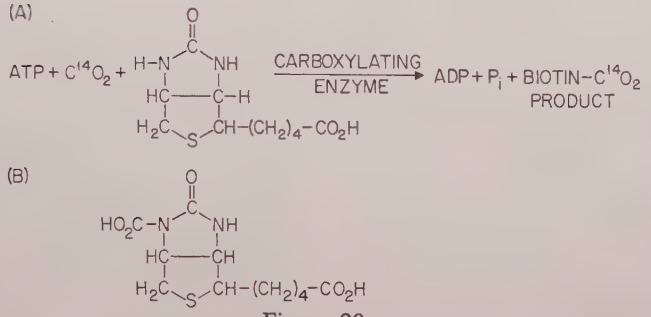


Figure 20

table thiol ester substrate to form the ive enol thiol ester. The activity of these P-activating enzymes, however, would absolutely dependent on the presence CO_2, which would presumably, in some y probably involving biotin (Lynen *et* '59), change the configuration of the zyme (Koshland, '58) so as to make it ive in its ATP-activating function. Such system, then, in which the primary nction of these activating enzymes is to oduce "active" ATP before its transfer the natural substrate might or might involve the reversible fission of the rophosphate linkages, depending on the ure of the activation process. It might s be possible to find examples involving ADP–ATP exchange as well as not.

The specific requirement of CO_2 for the ivation of this enzyme is understand- e in evolutionary terms when we exam- the nature of the proposed products med under the influence of this en- ne, namely, the phosphoenol thiol esters. These esters might be expected to be extremely labile to spontaneous hydrolysis if CO_2 were not present and if the enzyme remained fully active. This would, in effect, provide a fruitless mechanism of hydrolysis of the energy-storing compound, ATP. By requiring the mere presence of CO_2 to bring the enzyme to full activity, the true substrates for the carboxylation reaction of the enzyme must always be present when the primary product is formed, thus leading to the efficient use of the ATP.

Lynen has just presented evidence (this Symposium) for the formation of a compound between CO_2 and added free biotin under the influence of the enzyme for the carboxylation of β-methylcrotonyl—CoA and using ATP.[3] The properties of the products were briefly mentioned as including very great lability to dilute acid (*p*H 2) but considerable resistance to neutral or

[3] See footnote 2, page 60.

slightly alkaline media (*p*H 7–8) at ice temperatures (lifetime ~20 minutes) as evidenced by nonexchangeability of the $C^{14}O_2$ compound with nonradioactive CO_2 swept through the solution of the product of the enzymic reaction, as in figure 20A. The product was formulated as a carboxamidic acid (fig. 20B). This is almost certainly very unstable, having the carboxyl group free on an amide (urea) nitrogen atom. It is conceivable, however, that, as an anion, it would have the observed stability. Because the activity of the carboxylation enzyme in carboxylating β-methylcrotonyl—CoA depends on *bound* biotin, it was suggested that the "active" CO_2 in the enzyme has the same structure as that proposed for the free-biotin carboxylation product, and that the latter is formed in an exchange reaction with free biotin, according to the following sequence:

ATP: + biotin—enzyme ⇄
ADP—biotin—enzyme + P_i

ADP—biotin—enzyme + CO_2 ⇄
ADP + CO_2—biotin—enzyme

CO_2—biotin—enzyme + free biotin ⇄
CO_2—biotin (free) + biotin—enzyme
(isolated product)

If we accept the existence of such a free biotin–CO_2 compound, it is easy to conceive of its formation in terms of the enol carboxylation mechanism proposed as general in the body of this paper. The free biotin would be considered a *substitute* substrate for the enol phosphorylation and carboxylation (fig. 21).

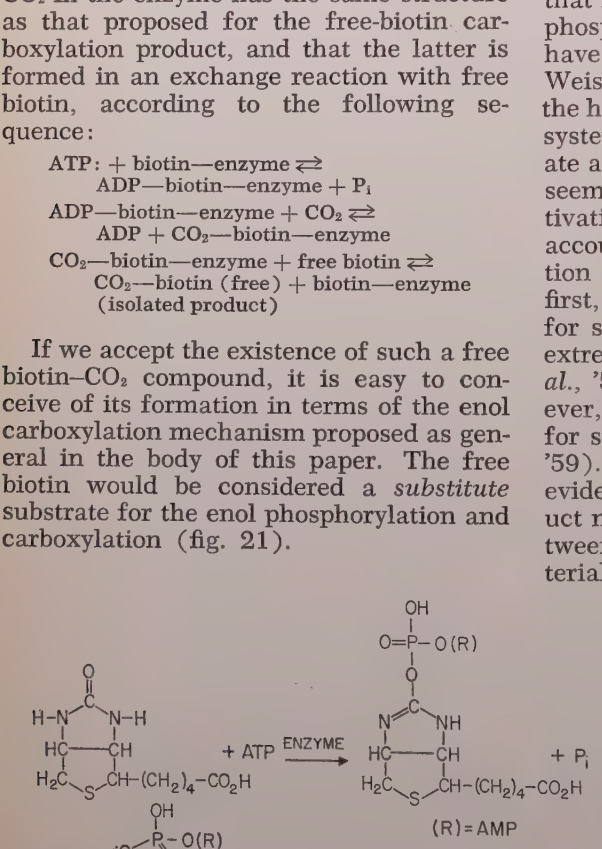
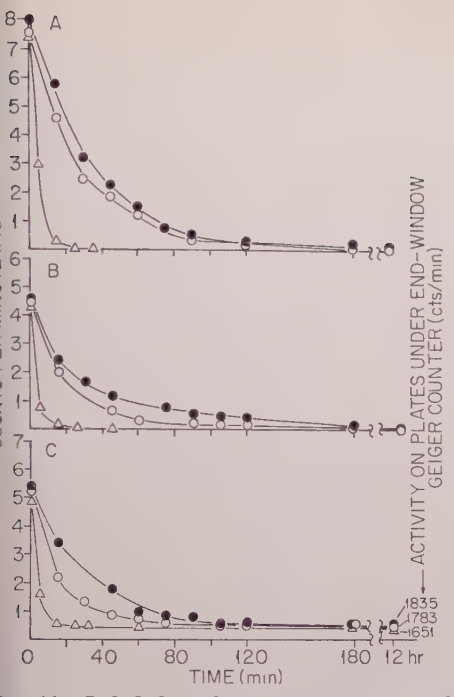


Figure 21

The fact that biotin is a much poo substrate for the disappearance of ATP u der the influence of this enzyme than its natural substrate, β-methylcrotonyl CoA, suggests that the "active" form either the CO_2 or the phosphate on the e zyme is not identical with that found the free biotin since this would involve relatively simple exchange reaction th might be expected to proceed rapidly. Th evidence, however, does not eliminate enzyme-bound biotin from direct implic tion in the CO_2-dependent ATP-activati function of the enzyme.

It seems that such a proposal as th accounts for the experimental observatio that have been reported and for which t phosphoric-carbonic anhydride syste have been devised. In the earlier work Weissbach *et al.* ('56) and Racker ('5 the high K_m values of the carboxydismuta system (when calculated on total carbo ate added, as observed in *in vitro* system seemed to require some form of CO_2 tivation in the *in vivo* systems in order account for the extremely rapid carboxy tion of ribulose diphosphate observed. first, there seemed to be some evider for such a CO_2 activation in the form extremely labile compounds (Metzner *al.*, '57, '58). Further investigation, ho ever, has failed to confirm any evider for such a product (Kasprzyk and Calv '59). In figures 22 and 23, we see that t evidence indicates the absence of any pr uct more stable than bicarbonate lying tween CO_2 and the relatively stable m terials that can withstand plating, e PGA. The chromatographic e dence for such a product h been accounted for otherw (Bassham *et al.*, '58).

However, an examination the *in vitro* carboxydismuta enzyme system revealed a pendence of the activity of t enzyme on the preliminary pr ence of CO_2 in addition to Mg Preliminary incubation of t enzyme with bicarbonate in t presence of Mg^{++} does inde induce a greater carboxylati activity than preliminary in bation with any other com



ig. 22 Labeled carbon in suspension after nol killing of algae. ●, Kept at —45°C.; ○, at —45°C., swept 15 minutes with N_2; △, at room temperature.

: Suspension of algae in 80% ethanol. One of 2% suspension of algae + 4 ml of ethanol, with 4 μc of C^{14} for 30 seconds and acidified 2 drops of glacial acetic acid.

: Algae kept in the dark, killed with ethanol. ml of 2% suspension of *Scenedesmus*, swept 1% CO_2 10 minutes and with N_2 3 minutes, of C^{14} added for 30 seconds in the dark. ed with 4 ml of acidified ethanol.

: Photosynthesizing algae killed with ethanol. ml of 2% suspension of *Scenedesmus*, pre-minated 10 minutes with 1% CO_2, swept 3 utes with N_2, 30 seconds' photosynthesis with of C^{14} (10 μl of 0.026 *N* $NaHC^{14}O_3$), killed 4 ml of acidified ethanol.

t of the reaction system (see table 2 figs. 24 and 25) (Pon, '59).

is perhaps worth noting at this point the precise investigations of the kine- of carbon flow in the *in vivo* systems ugh the carboxydismutase reaction n to indicate that, whereas in the dark primary product of carboxylation does ed split into two molecules of PGA, he light it may be otherwise. In the t there is an indication that the primary luct of carboxylation may be splitting reductive reaction, leading to only one molecule of PGA and one molecule at the triose phosphate oxidation level (Bassham, '59).

Such an alternative, of two possible modes of action, was proposed when the carboxylation of ribulose diphosphate was first recognized (Calvin and Massini, '52; Wilson and Calvin, '55; Bassham and Calvin, '57). The best evidence that it might be so has only recently been found in more-precise measurements of the rate of approach to C^{14} saturation of the pools of

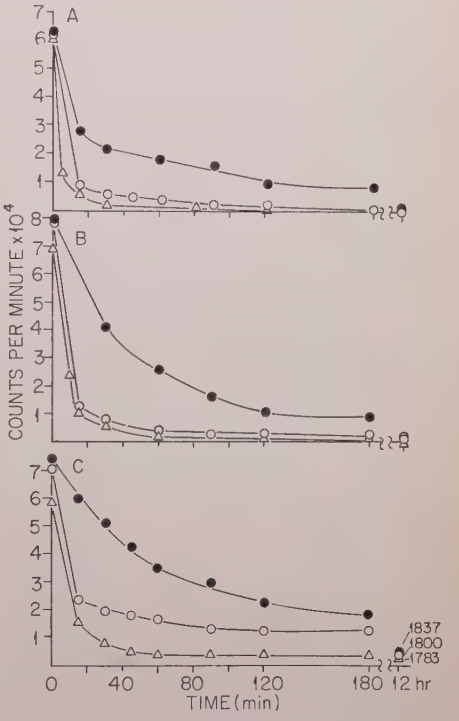


Fig. 23 Labeled carbon in suspension after acetone killing of algae. ●, Kept at —45°C.; ○, kept at —45°C., swept 15 minutes with N_2; △, kept at room temperature.

A: Suspension of algae in 90% acetone. One ml of 4% suspension of *Scenedesmus* + 9 ml of acetone, kept with 4 μc of C^{14} for 30 seconds and acidified with 4 drops of glacial acetic acid.

B: Algae kept in the dark, killed with acetone. One ml of 4% suspension of *Scenedesmus* swept with 1% CO_2 10 minutes, and with N_2 3 minutes, afterward 4 μc of C^{14} added for 30 seconds in the dark. Killed with 9 ml of acidified acetone.

C: Photosynthesizing algae killed with acetone. One ml of 4% suspension of *Scenedesmus* pre-illuminated 10 minutes with 1% CO_2, swept 3 minutes with N_2, 30 seconds' photosynthesis with 10 μc of C^{14}, killed with 9 ml of acidified acetone.

TABLE 2

Preincubation of carboxydismutase with substrates and cofactor

	Total counts/min.		Total counts/
E + M → EM → EMS_1 → PGA (via S_2)	24,000	M + S_1 → MS_1 → MS_1E → PGA (via S_2)	2
E + M → EM → EMS_2 → PGA (via S_1)	13,000	M + S_1 → MS_1 → MS_1S_2 → PGA (via E)	1
E + S_1 → ES_1 → ES_1M → PGA (via S_2)	24,000	M + S_2 → MS_2 → MS_2E → PGA (via S_1)	1
E + S_1 → ES_1 → ES_1S_2 → PGA (via M)	13,000	M + S_2 → MS_2 → MS_2S_1 → PGA (via E)	1
E + S_2 → ES_2 → ES_2S_1 → PGA (via M)	9,400	S_1 + S_2 → S_1S_2 → S_1S_2E → PGA (via M)	1
E + S_2 → ES_2 → ES_2M → PGA (via S_1)	10,000	S_1 + S_2 → S_1S_2 → S_1S_2M → PGA (via E)	1
1st: PREINCUBATION (each 10 min, 0°C); 2nd: INCUBATION (5 min, 25°C)		1st: PREINCUBATION (each 10 min, 0°C); 2nd: INCUBATION (5 min, 25°C)	

E, Carboxydismutase; S_1, $NaHC^{14}O_3$; S_2, ribulose 1,5-diphosphate; M, Mg^{++}; PGA, 3-phospho ceric acid-1-C^{14}.

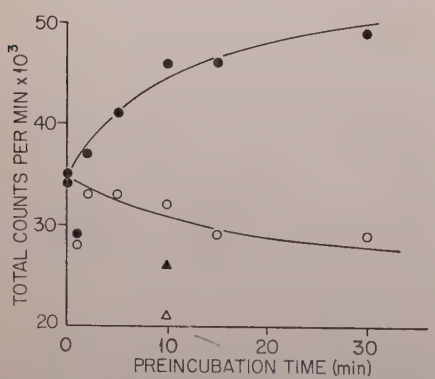


Fig. 24 The preincubation of carboxydismutase with substrates at 0°C. ●, Enzyme + Mg^{++} (0.01 *M*) preincubated with $HC^{14}O_3^-$ (0.0067 *M*); ○, enzyme + Mg^{++} preincubated with RuDP ($\sim 5 \times 10^{-5}$ *M*); ▲, enzyme preincubated with $HC^{14}O_3^-$; △, enzyme preincubated with RuDP. All incubations at 25°C., 5 minutes.

PGA and ribulose diphosphate in algae and in a more nearly true steady state of photosynthesis than has heretofore been achieved (see fig. 26). From this it is possible to show that only if a single molecule of PGA is liberated for each molecule of CO_2 entering the algae d the specific activity (C^{14}) of the ribul diphosphate remain higher than that the α- and β-carbon atoms of PGA. remaining three carbon atoms in the action go directly to the sugar level oxidation when the light is on.

Two possible sequences fulfilling th requirements are shown in figure 27.

Much remains to be accomplished bef we will know the intimate details of t

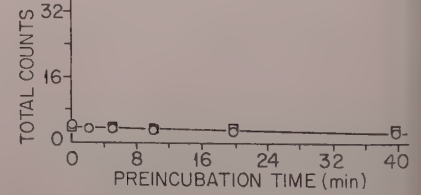
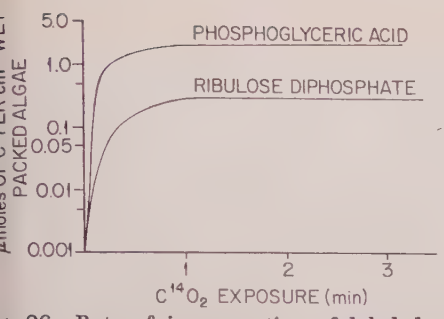


Fig. 25 Preincubation of "aged" lyophil carboxydismutase with various metal ions. 0.01 *M* Mg^{++}; □, 0.01 *M* Ni^{++}; ○, 0.0 Co^{++} and 0.01 *M* Mn^{++}.



g. 26 Rate of incorporation of labeled car- from labeled bicarbonate into phosphoglyceric by ribulose diphosphate. $C^* = C^{12} + C^{14}$ as $^{14}O_2$ administered.

tion, and it is not impossible to con- e that the reductive splitting of the in- ediate carboxylation product (β- or to acid) might very well require a re- ing system as yet unknown.

OPEN DISCUSSION

AGER[4]: In connection with the postu- d enol intermediate involved in the car- ylation reactions discussed by Dr. Cal- vin, I thought that I might briefly describe a decarboxylation reaction that fits into this general category and leads to the formation of a C—chloride bond. This work arose out of our interest in the biosynthesis of some of the microbial metabolites containing chlorine. In the biosynthesis of caldariomycin (2,2-dichloro-1,3-cyclopentanediol) isotopic evidence indicates that δ-chlorolevulinic acid is the first chlorinated intermediate, which later, through a series of reactions, eventually is cyclized to form the cyclopentane ring. In enzymic studies of the formation of δ-chlorolevulinic acid, it was possible to obtain a soluble enzyme preparation capable of catalyzing the formation of δ-chlorolevulinic acid from β-ketoadipic acid and chloride ion. The stoichiometry of the reaction indicates that 1 mole of β-ketoadipic acid reacts with 1 mole of chloride ion and 0.5 mole of oxygen to form 1 mole of δ-chlorolevulinic acid plus CO_2 according to figure 28.

[4] L. P. Hager, Harvard University.

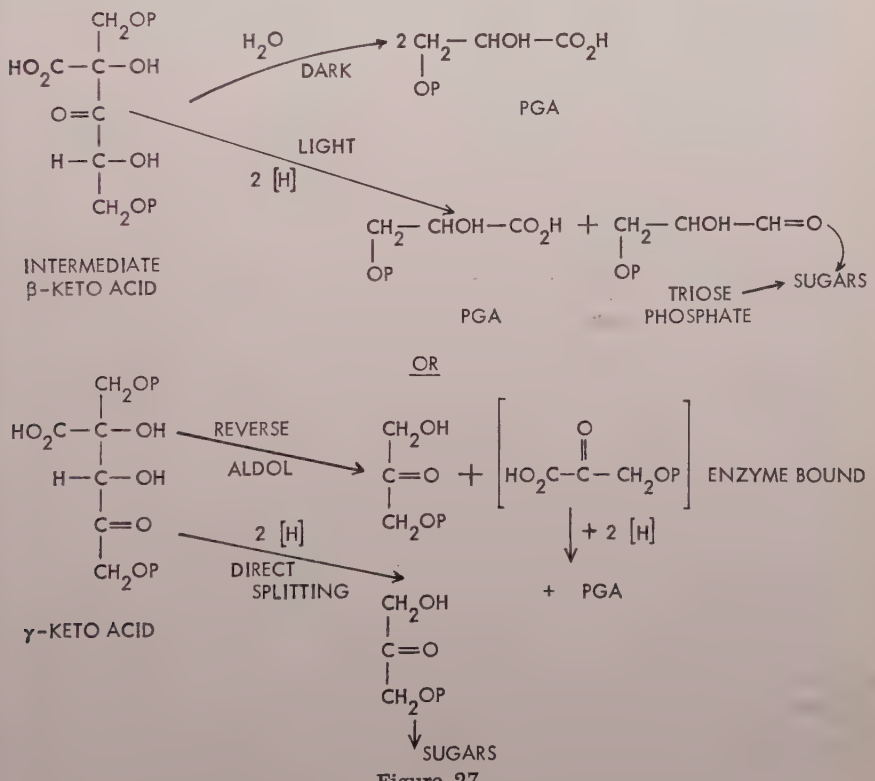


Figure 27

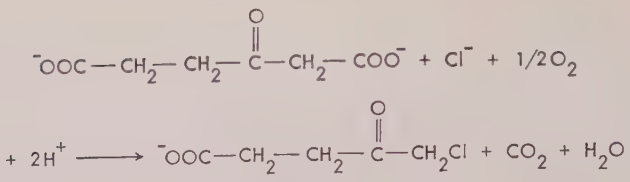


Figure 28

The mechanism, which was previously discussed for the decarboxylation of β-keto acids in general, involving the formation of the intermediate enol, suggests that a mechanism for this chlorination reaction might involve first the decarboxylation of the β-keto acid to yield an enzyme-bound enol form of levulinic acid. By analogy to chemical halogenation, a stabilized chlorinium ion (enzyme-bound or prosthetic-bound form of chlorinium ion) formed by oxidative removal of two electrons from chloride ion would then probably serve as the active chlorinating agent for the formation of δ-chlorolevulinic acid.

JONES[5]: I should like to say a few words about C—N bonds that involve CO_2 or more correctly bicarbonate fixation; namely, the mammalian fixation of bicarbonate into carbamyl phosphate. We believe that the first two steps in carbamyl phosphate synthesis with mammalian (or frog) enzymes are the same as those Dr. Lynen described for the formation of biotin-CO_2. We have not been so fortunate as to find a base-stable CO_2 compound, as Dr. Lynen has, although we believe such a compound does exist. In carbamyl phosphate synthesis, however, biotin does not seem to be involved; acetyl glutamate performs the analogous function. The fact that an acyl glutamate was required as cofactor was demonstrated very early by S. Grisolia and P. P. Cohen. The structures of biotin and acetyl glutamate are shown in figure 29 and you can readily see (the dotted line) that they have one grouping in common—a substituted amide group—which we believe is the functional group in carbamyl phosphate synthesis. Hydroxylamine in this system has an unusual effect. From our earlier studies carried out in Dr. Lipmann's laboratory, we knew that hydroxylamine did not react rapidly at 37°C. with carbamyl phosphate, as it does with acetyl phosphate, to yield hydroxamate.

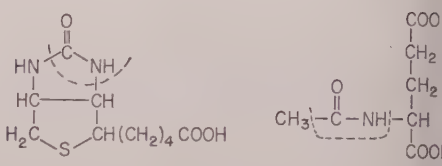


Figure 29

Therefore, when we reconfirmed the droxylamine-stimulated ATPase, first served by S. Grisolia and R. O. Mars in this reaction, we believed that it doing something other than reacting carbamyl phosphate. Recent studies paring ammonium, hydrazine, and droxylamine have convinced us that droxylamine is not replacing ammoni form the *N*-hydroxycarbamyl phosph Since the ATPase activity requires hydroxylamine and bicarbonate and other phosphate compounds than P_i ADP accumulate, we think that hydr amine splits a bicarbonate-acetyl gl mate intermediate. Dr. Lynen and I discussed this, and he promises that is going to try hydroxylamine on his biotin compound.

Dr. Leonard Spector has synthes acetyl glutamate with O^{18} in the ac carbonyl group to test whether this gen participates in the formation of intermediate (either phosphate, ad diphosphate, or even bicarbonate). we are looking for a loss of O^{18} and though the loss observed in the incuba is at present only 15%, we think that is because only 1 mole of acetyl glutar is bound to the enzyme and recycled. are rather certain that this loss is nificant since our control vessels have sistently shown no loss. We are now c ing out more-sensitive studies, workin this hypothesis to see if we can incr the loss. When that condition is obta we will obviously have to look for

[5] M. E. Jones, Brandeis University.

poration of the O^{18} into one of the phate compounds to make the obser- n meaningful.

ke Dr. Lynen, we have done P^{32}-phos- e-exchange studies, and we, too, find his exchange occurs when the enzyme cubated with ADP and P^{32}. Actually, et some exchange without the addition ıy acetyl glutamate, although the ex- ge is increased twofold by the addition etyl glutamate. The dramatic finding, ver, is that the addition of bicarbonate this exchange to zero; so that there possibility that we have an ADP in of a P_i intermediate. I think the O^{18} es once again will help to decide this .

EENBERG[6]: I should like to take the ion of talking about another person's . Since I am not qualified you will to forgive me, but Dr. M. J. Coon's has been brought up here in several and I think perhaps I can put it in a sympathetic light as the result of work that he will report at the Feder- meetings.

st I might point out that Coon and oworkers reported at the Ciba con- ce a year ago that they were unable peat the studies of B. K. Bachhawat Coon. This is a system in which an ne had been crystallized and that ently gave AMP and pyrophosphate. they have studied this in much more , and this reaction, which I am un- fied to say is concerned with car- ation—I think probably it is not— ves ATP, hydroxylamine, CO_2, a di- t metal (zinc appears to be the most e), and this crystalline enzyme. The ıct of this reaction is ADP and a ound that has the properties of hy- lamine phosphate. This hydroxyl- e phosphate (I must say this very ully because I am talking about some- else's work) has been isolated. It s down to 1 mole of hydroxylamine mole of phosphate and contains no active CO_2. It does have impurities, uld add. The ADP in this system nges with ATP but AMP does not, he reaction is CO_2 dependent.

ROMINGER[7]: I should like to compare eactions of phosphopyruvate and the reactions of CoA derivatives, and then to ask a question. First, there is either phosphoryl or CoA transfer, phosphoenolpyruvate kinase, and various thiophorases. Second, there are acyl transfer reactions. Enolyl pyruvate is transferred in the synthesis of uridinediphosphoacetylglucosamine pyruvate. Another reaction of this kind has been found by Davis in aromatic ring synthesis. Phosphotransacetylase would be an example of an acyl transfer reaction of a CoA derivative. Reversible hydration reactions occur with both types of compounds, e.g., enolase and the hydration-dehydration of α,β unsaturated CoA derivatives.

Then there are the condensation reactions on the methylene carbon: phosphoenolpyruvate condensation with erythrose 4-phosphate or the condensation of acetyl —CoA with oxaloacetate in citrate formation. Finally, both of the speakers mentioned the CO_2 fixation reactions of phosphopyruvate (oxaloacetic carboxylase). However, the analogous reaction of acyl—CoA compound with CO_2 to give a carboxylated compound plus free CoA has not been found. Is there any chemical reason why such a reaction might not occur?

CALVIN: Well, isn't ATP required for some of these on the CoA side? It certainly is required for the carboxylation of acyl—CoA.

STROMINGER: No ATP would be required in the reaction I am suggesting.

STADTMAN[8]: He said that the last one does not occur.

STROMINGER: That would be the carboxylation of the acyl—CoA compound with the simultaneous cleavage of the thioester.

CALVIN: The way I had it formulated it was a simultaneous cleavage of the phosphate. You see, as the carboxylation goes the phosphate comes off. It is just like the enol phosphate. That is all.

TODD[9]: I am most interested and pleased to hear Dr. Calvin's views on these mat-

[6] G. R. Greenberg, University of Michigan.
[7] Jack Strominger, Washington University, St. Louis.
[8] E. R. Stadtman, National Institutes of Health.
[9] Alexander Todd, University Chemical Laboratory, Cambridge, England.

ters, because oddly enough we in Cambridge have been thinking about some of the same problems and reached much the same conclusions. We know that the enol phosphate derived from diethyl malonate is quite an active phosphorylating agent, and we know that, with less highly activated enol phosphates such as phosphoenol pyruvate, if you remove a couple of electrons from the system, the phosphate at once comes off. There is actually a very close analogy between phosphorylation and carboxylation reactions. If we think of the conversion of phosphoenol pyruvate to oxaloacetate, then if the positive CO_2 attacks the doubly bound CH_2 in the phosphoenol pyruvate, the effect will be similar to that produced by removal of electrons in an enol phosphate, or protonation of an imidoyl phosphate. In other words, phosphate will be expelled and the CO_2 will become attached, yielding oxaloacetate.

CALVIN: Also phosphorylation.

TODD: Yes, you get carboxylation and phosphorylation linked together.

Perhaps I might just mention in connection with Professor Lynen's earlier point about the fascinating things he has been finding with isopentenyl pyrophosphate —that C—C bond formation of the type he was discussing was done using phosphates some years ago in the laboratory. We have used the alkylation reaction of phosphates a great deal in connection with partial debenzylation of phosphates. Now benzyl and allyl are rather similar groups, although we have not used allyl compounds a great deal merely because they are more inconvenient to handle than the benzyl compounds. But one method of debenzylation that we have used is to treat a benzyl phosphate with phenol. When this is done, the benzyl group is removed as ortho- and parabenzyl phenol.

This reaction with phenol is, of course, formally rather like the reaction of an olefin, and I think you may be interested to know that some time ago Dr. F. R. Atherton, a former colleague of mine, found that geranyldiphenyl phosphate, which he synthesized, underwent a change on standing, diphenyl phosphate being ejected and a mixture of cyclic monoterpene and higher polymeric material being produced. T is, of course, strictly analogous to the synthetic processes discussed today, I would only make the point that I my do not believe that actual ionization of allylic phosphates before attack of the fin ever occurs; attack and expulsion the phosphate, or, for that matter, p phosphate, will be simultaneous. So th is a straight laboratory analogy for reactions Dr. Lynen was talking ab and his postulated reactions are there entirely reasonable.

LYNEN[10]: From the chemical point view I am very pleased to hear that kind of polymerization also occurs n enzymically.

TODD: I think it is just a point interest to mention, particularly since observations were made in the course work with an entirely different aim.

LYNEN: That is interesting. In case, which double bond of the gera derivative reacts with the allylic grou the other?

TODD: It is the double bond at the o end of the molecule.

LYNEN: You would then expect the mation of a quaternary carbon atom.

TODD: But the double bond can n and cyclization can therefore occur q readily. The double bond is not in allylic position. This is a simple do bond, but I agree that it is so placed s cally in relation to the diphenylphosp ester group that reaction to cyclize is ored, but I would stress that there mixture of products and not only simple monocyclic terpene.

LYNEN: We have not yet establis whether pyrophosphate is released in enzymic polymerization reaction or haps inorganic phosphate in analog the alkylation of methionine by the ad sine moiety of ATP. The driving forc this type of alkylation would seem t derived from the splitting of a pyrop phate bond. One could visualize meth ine at the active site of the enzyme a ally participating in a transalkylation the formation of an "allyl sulfonium" zyme—substrate intermediate.

[10] Feodor Lynen, Max-Planck-Institut für chemie.

ODD: In that case I think that the phosphate would probably be sufficient. This is, of course, just a guess based the strength of pyrophosphoric acid, ch I think would be sufficient for the pose, although there may be this other rmediate to which you have referred.

ARSON[11]: If it will give Harland Wood comfort, he ought to know that we e confirmed his experiments on the e type of exchange and have had ex- y the same difficulty.

TERN[12]: Because of the interest that been generated in malonyl—CoA ce it has been demonstrated to be ned by the carboxylation of acetyl—), I should like to mention other en- ic mechanisms by which malonyl— can be synthesized. The original one he activation of malonate via an ATP-, -dependent reaction to malonyl—CoA, ch was first described in malonate- pted *Pseudomonas* by O. Hayaishi. The urrence of this reaction in rat kidney shown by H. I. Nakada *et al.* and firmed by R. O. Brady; we also found ccurring in heart and muscle tissues. More recently Dr. Menon and I found ther biosynthetic route for malonyl— A via a CoA-transferase type reaction in ich the donor is acetoacetyl—CoA. In dying CoA transfer reactions in dog skel- muscle extract, we found that aceto- tyl—CoA transfers CoA to malonate, cinate, and glutarate to form the cor- ponding monoacyl—CoA of the dicar- ylic acid. Some years ago in the New k laboratory, we purified from pig heart enzyme we called succinyl-β-ketoacyl— A transferase that transfers CoA from toacetyl—CoA to succinate. During the ification of this enzyme from dog skele- muscle extract, we found that there s a constant ratio of the reactivity of lonate and succinate as acceptors for A from acetoacetyl—CoA and that this io was 1:50. We therefore went back the 6-year-old preparations of the pure heart CoA transferase and found that too, transferred CoA from the aceto- tyl—CoA to malonate. In our paper we d that malonate was unreactive, be- use we did not appreciate that the factor reactivity would be only one-fiftieth that succinate. It is possible to use CoA transferase to biosynthesize malonyl—CoA by the reaction of acetoacetyl—CoA and malonate since the equilibrium favors malonyl—CoA formation. Biosynthetic malonyl—CoA is converted to acetyl—CoA in pigeon liver extracts as shown by coupling with oxaloacetate and measuring citric acid synthesis. Dr. Lynen has also mentioned this conversion.

Another interesting aspect is that CoA transferase will also effect malonyl—CoA synthesis from succinyl—CoA and malonate. This reaction provides a link between the citric acid cycle and the fatty acid synthesis. I might say that I have learned from Dr. D. E. Green that Dr. S. J. Wakil, at Wisconsin, has been synthesizing malonyl—CoA by this type of exchange reaction.

STADTMAN: I would like to mention briefly some studies being carried out in our laboratory by Dr. P. R. Vagelos that are relevant to the question of malonyl—CoA synthesis and the mechanism of active CO_2 formation. Dr. Vagelos has found that cell-free extracts of *Clostridium kluyveri* catalyze the oxidation of propionyl—CoA. This is a β oxidation process involving the intermediary formation of acrylyl—CoA, which is hydrated to form β-hydroxypropionyl—CoA. The β-hydroxypropionyl—CoA is then oxidized by successive TPN-linked dehydrogenation reactions to malonyl—semialdehyde—CoA and to malonyl—CoA. Although the latter compound does not seem to undergo extensive enzymic decarboxylation to acetyl—CoA and CO_2, an enzyme system is present that catalyzes the rapid equilibration of $C^{14}O_2$ with the carboxyl group of malonyl—CoA. This exchange reaction is of further interest because, with purified enzyme fractions, it is absolutely dependent on the presence of a thermal, stable cofactor derived from boiled extracts of *C. kluyveri.* The heat-stable cofactor cannot be replaced with biotin nor is the exchange reaction inhibited by avidin. ATP and other obvious nucleotide derivatives are without effect on this system.

LYNEN: May I suggest a possible mechanism for this exchange. If malonyl—CoA

[11] S. F. Carson, Oak Ridge National Laboratory.
[12] J. R. Stern, Western Reserve University.

does indeed condense with itself, acetone dicarboxylyl—CoA and CO_2 would be formed. If this reaction were reversible, labeled CO_2 would be incorporated into malonyl—CoA.

STADTMAN: What would be the role of the coenzyme?

LYNEN: I don't know. It is merely speculation.

STADTMAN: There is one other point that I did not mention. That is, the system is very markedly activated by acetyl—CoA. So the way we would visualize the reaction formally is that malonyl—CoA is cleaved to acetyl—CoA plus CO_2X; the latter in turn can equilibrate with the CO_2.

KOSHLAND[13]: Since Dr. Lynen was willing to speculate, I will follow his lead with a mechanism that might possibly explain the confusing isotopic data of some of these coenzyme reactions. Thus, in the case of the carboxylate activation via a CoA-phosphate intermediate, the sequence of reactions shown in figure 30 might occur. In the first step ADP is displaced by CoA, in the second step the CoA is displaced by the carboxyl group, and in the last step the phosphate ion is displaced by CoA. Why nature should choose this roundabout mechanism is not clear, but it is at least consistent with the isotopic data and the intermediates obtained by I. C. Gunsalus, M. Cohn, and others, whereas the straightforward displacement of carboxyl on ATP is not. Moreover, there is analogy for this type of sequential shifting of bonds in the molecular rearrangements of organic chemistry. The last two steps might be considered an enzymic molecular rearrangement that proceeds rapidly because the —SH of CoA is liberated in the immediate vicinity of the tivated carboxyl group.

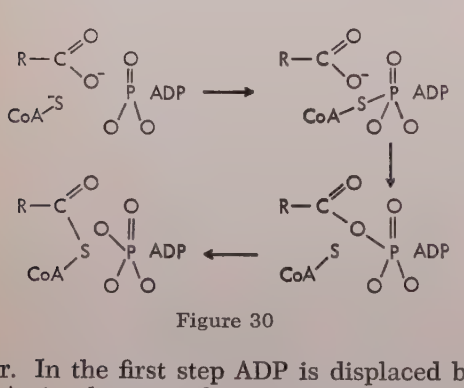


Figure 30

[13] D. E. Koshland, Brookhaven National Labo tory.

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ıino Acid Activation and Protein Synthesis[1]

FRITZ LIPMANN, W. C. HÜLSMANN, G. HARTMANN,
HANS G. BOMAN, AND GEORGE ACS
The Rockefeller Institute, New York City

Ve have been talking all day about ynthetic mechanisms. A great deal of discussion has concerned what many s feel is one of the most important as- s of a biosynthetic problem, namely, ecognize the activated compound that s into a synthesis. What kind of com- nd is it? In what form is it activated so it can become part of a growing unit, ut it very generally?

Vhen I first started to think about pro- synthesis, I was rather naive about it thought it would be just another prob- in biosynthesis. Obviously, a peptide has to be formed, and I hoped that if could only find out how the amino acid ctivated to form such a link, we would w a great deal about protein synthesis ɔmann, '49). In due time, I have come ealize that this was a very wrong no- . It has become clear to many of us protein synthesis, although it seem- y presents a relatively simple problem iosynthesis—that of joining a carboxyl up to an amino group by dehydration— , in fact, a very formidable one because tide linking is a premise only. The real blem is to find out how the amino acids induced to link in just the right order epresent the specific quality of a par- lar protein. I still feel that we are e far removed from a profound under- ıding of this biologically fundamental blem. We have to visualize that even relatively small protein such as ribo- lease (Hirs *et al.*, '56), we are dealing ı a sequence of 124 amino acids that d to be arranged in the right order.

AMINO ACID ACTIVATION: FIRST STEP

ı my earlier work, I have dealt with formation of peptidic bonds on several ıasions. When I was interested in the ylation problem, a test system in which acetylation of aromatic amines was measured proved to be very helpful (Lipmann, '45), and acetylation of aromatic amines is a formation of a peptidic bond. Some types of peptidic bond, such as the above-mentioned and also that in hippuric acid (Chantrenne, '51) appeared to be formed by way of CoA, and for a while aminoacyl —CoA, or more generally an aminoacyl thioester, was thought to be the active amino acid in protein synthesis. However, that turned out not to be so.

Other processes of peptide linking were found. For example, the synthesis of glutathione was elaborated by Bloch and his collaborators (Yanari *et al.*, '53). Here, the terminal phosphate of ATP is the energy carrier in the activation process, as it is also in the process of glutamination (Speck, '49). So far, however, all these reactions seemed unfit for protein synthesis because they did not show a generality for all amino acids as one would expect of a reaction that is truly involved in protein synthesis.

In the course of our studies on the transfer of energy from ATP to form acetyl—CoA (Lipmann *et al.*, '53), we stumbled unexpectedly on a liberation of pyrophosphate, instead of what we had tacitly always expected to be phosphate:

$$\text{Acetate} + \text{AP} \sim \text{PP} + \text{CoASH} \rightleftharpoons \text{acetyl} \sim \text{S.CoA} + \text{PP}$$

This impressed us then as being a rather unusual reaction in this kind of process and, at first, we did not understand it correctly. But Paul Berg ('56) recognized that it was attributable to the initial formation of acetyl adenylate as an intermediate. From then on, we became aware that

[1] This work was supported by grants from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service, and from the National Science Foundation.

whenever there is such a pyrophosphate liberation, an acyl adenylate formation should be expected:

$$\text{Acylate} + \text{AP} \sim \text{PP} \rightleftharpoons \text{AP} \sim \text{acylate} + \text{PP}$$

During that period, Maas ('53) found that the peptidic link in pantothenic acid was formed through pyrophosphate displacement in ATP. In view of previous failures to discover generality with the mentioned enzymic possibilities of peptide bond formation, we hoped that this new one involving pyrophosphate liberation might be right for general amino acid activation. This idea was incorporated in a scheme that, although quite vague in details, served for a while as a skeleton for discussing the actual problems involved in the synthesis of a protein (Lipmann, '54). Most important, however, was the fact that it induced Mahlon Hoagland ('55) to try for such an amino acid activation in the Zamecnik system of amino acid incorporation into protein; and, as you all know, this led to the discovery of the presence of enzymes of this type in liver homogenate, or more precisely, in the 100,000 × *g* supernatant of rat liver homogenate (Hoagland *et al.*, '56). With this discovery, the gate was opened toward a more precise and more profound understanding of the process of amino acid activation. This will be the main topic of my paper.

Hoagland found activation of a fair number of amino acids in liver extract. However, it still is rather difficult to show that all amino acids are activated in this manner. A complete set of activation is presented in table 1, when PP exchange was used as assay. The excess PP exchange is measured here, which appears on addition of a particular amino acid and which we find to be not only a more sensitive but also a more reliable assay. Such a complete response was found in pigeon pancreas supernatant (Lipmann, '58) and also in a microbial preparation (Nissman *et al.*, '57). As can be seen in table 1, the excess exchange was small with some amino acids. Glutamic acid was one of the most difficult to show, but in one case at least, exchange was well above the background. In spite of some disagreement on this point, we feel justified in concluding from such experiments that we dealing with a general activation proc

TABLE 1

Amino acid-dependent PP^{32}-ATP exchange pigeon pancreas extract

(Lipmann, '58)

Amino acid	mμmoles of PP^{32} exchanged per mg of protein[a]
Arginine	2.3(1–3)
Glutamic	4.0(0–9)
Glutamine	4.4
Asparagine	8.1
Glycine	12.0
Methionine	19.0
Phenylalanine	26.0
Isoleucine	26.0
Serine	32.0
Leucine	35.0
Lysine	44.0
Cysteine	46.0
Aspartic	53.0
Tyrosine	55.0
Alanine	58.0
Valine	65.0
Tryptophan	105.0
Threonine	108.0
Histidine	142.0
Proline	179.0

[a] Every figure represents an average of experiments. A background exchange, wi amino acids, of 18–20 mμmoles of PP^{32} per m protein was deducted in all cases.

The similarity of the initial step amino acid activation to that in ace activation was proved by Novelli and group (DeMoss *et al.*, '56). They were first to synthesize leucyl adenylate could show that it reacted as expec namely, that on addition of PP it for ATP, arriving thus at the formulation

$$\text{AP} \sim \text{PP} + \text{amino acylate} \rightleftharpoons \text{AP} \sim \text{amino acylate} + \text{PP}$$

They showed the presence of these zymes in many bacterial extracts (De and Novelli, '55), and Webster found t in plants ('59). Berg ('58), using methionine-activating enzyme from m bial sources, studied the reversible reac in greater detail.

At first, it seemed possible that t might be one, or only a few relatively specific enzymes. But the early wor Hoagland *et al.* ('56) already mad more likely that there was a special zyme for every amino acid. The speci question was partly clarified when H *et al.* ('56) isolated the tryptophan-act

enzyme from beef pancreas, in which s very abundant, and obtained a nearly nogenous tryptophan-specific enzyme paration. This indicated, by implica-n, that the activating enzymes were ino acid specific. In the meantime, a mber of activating enzymes have been ated by Berg ('58) and Schweet and en ('58).

AMINO ACID ACTIVATION: SECOND STEP

Nevertheless, all during the earlier work activation of amino acids, it was ner obvious that, although the product s aminoacyl adenylate, no measurable ounts of it could be detected free in ution. It was only when large enough ounts of tryptophan-activating enzyme re available that stoichiometric amounts ld be used did Meister's group (Karasek *al.*, '58) and Davie's group (Kingdon *et* '58) analytically establish a formation tryptophan adenylate. In particular, vie and his group at Western Reserve wed that the amount of intermediate s rather exactly equivalent to the ount of enzyme. Therefore, although mechanism of the reaction appeared it, we seemed to understand the first f only. The ultimate acceptor for the ino acid was still missing.

The solution to the mystery came from k by Hoagland, Zamecnik, and Ste-nson ('57), who demonstrated that the vated amino acid was transferred to a ble ribonucleic acid (s-RNA) and was ed to it in a relatively unstable linkage. about the same time, other laboratories orted indications of an intermediate . Hultin and Beskow ('57) and Holley ') showed indirectly that there was bably a cytoplasmic mediator between activating step and the microsome. Our rest was aroused to try and understand chemical linkage by which the amino l was attached to RNA. Early observa-s indicated to us that the aminoacyl–NA link was chemically too stable to be rboxyphosphoanhydride. This more or excluded the fact that amino acids be attached to a bridge phosphate, be-se a link between a carboxyl and the remaining hydroxyl of a doubly sub-uted phosphate was expected to be particularly unstable. There was still the possibility of a linkage to a terminal phosphate, which might be qualitatively similar to aminoacyl adenylate. This seemed to be excluded by a comparison between the latter's stability and that of the amino acid link to RNA. When hydroxylamine was used at low temperature and at a *p*H relatively unfavorable to hydroxamate formation as a sensitive test system, the stability of the aminoacyl–RNA, as shown in figure 1, resembled an aminoacyl ester

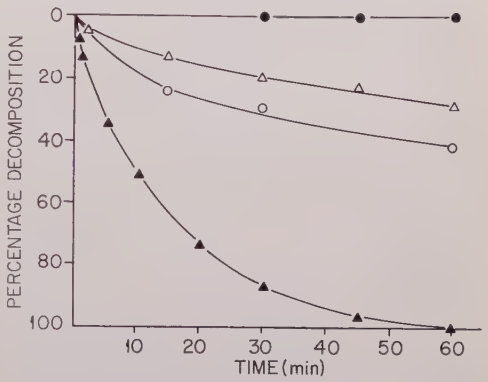


Fig. 1 Reaction of amino acid derivatives with 1 *M* hydroxylamine at *p*H 5.5 and 0°C. (see table 1 in Zachau *et al.*, '58). The leucine ester of AMP was prepared according to Wieland *et al.* ('57) and the leucyl-AMP anhydride by a modification of the procedure of Berg ('57). With the synthetic compounds hydroxamate formation was measured (Lipmann and Tuttle, '45). With C^{14}-leucyl-RNA, the liberation of radioactivity was followed. ●, Leucine ethyl ester; △, 2′ or 3′ leucine ester of AMP; ○, C^{14}-leucyl-RNA; ▲, leucyl-AMP anhydride.

(Raacke, '58), particularly the ester of leucine carboxyl with the 2′ or 3′ hydroxyl of the ribose in adenylic acid (Zachau *et al.*, '58). In contrast, the mixed anhydride of leucine and adenylate reacted much more rapidly.

By good luck, Drs. Zachau and Acs and I ('58) could relatively easily confirm this suspicion that the amino acid was esterified to the 2′ or 3′ position of a ribose in the RNA. We subjected a ribonuclease digest of amino acid-charged s-RNA, which was also charged with radioactive leucine as a marker, to paper electrophoresis at *p*H 3–3.5. As can be seen in figure 2, all radioactivity migrated fairly rapidly toward the negative and was therefore attached to a positively charged fragment. The

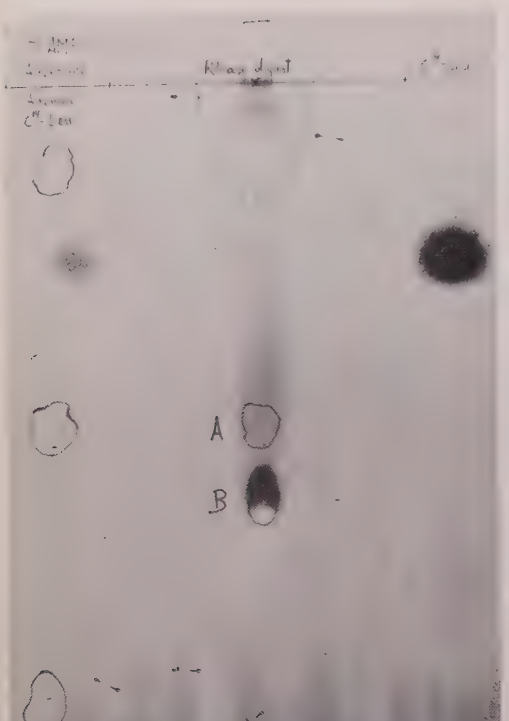


Fig. 2 Electrophoresis pattern of RNase digest of C^{14}-leucine-labeled s-RNA (Zachau *et al.*, '58).

radioactivity appears on the electropherogram in the lower spot and coincides with ultraviolet quenching, indicating the presence of a nucleoside in view of the fast migration toward the negative. The upper spot just above the radioactive one showed quenching only.

It was most significant that the radioactive spot containing the amino acid-carrying material did not give periodate reaction, whereas the slower material just above it was periodate positive and turned out to be largely free adenosine. In the eluate of the faster moving, partly radioactive spot, the ultraviolet-absorbing material was also identified as adenosine, but bound to ninhydrin-reactive material present in rather closely equivalent amounts, of which radioactive leucine accounted for only about 4%. This indicated that the faster moving area contained a mixture of amino acids linked to adenosine.

From these data, it was concluded th the amino acids were transferred from t initial activation product, namely amin acyl adenylate, to terminal adenosine s-RNA. The absence of periodate reacti in the adenosine derivative indicated blo ing of one of the adjacent 2′,3′ hydrox and the equivalence of ninhydrin-react material, liberated by mild alkali, a adenosine indicated a linkage of ami acids to the 2′ or 3′ position. We belie that biochemically active compounds ca amino acids *either* in the 2′ *or* 3′ positi Which of the two, however, is the po of attachment is open to guesswork o at present. Figure 3 shows a reconstr

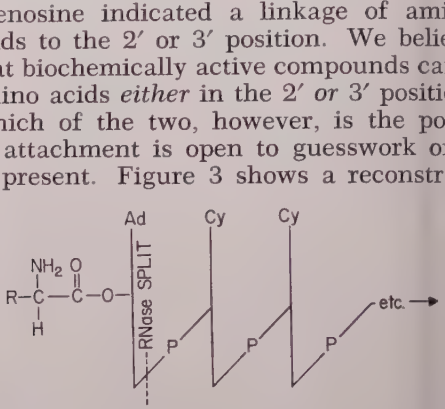


Fig. 3 Formulation of the linking of an acid to the terminal adenosine of s-RNA.

tion from analysis of the RNase digest the terminal RNA with an amino acid tachment. At the dotted line, RNase expected to split off the terminal adenos from neighboring cytidylic acid. The minal sequence in s-RNA is cytidylic tidylic-adenylic, as has been previously vealed through work in various labora ies, and most recently by Hecht *et al.* ('5

From the discussed analysis of the e trophoresis pattern, it appeared that un our conditions, amino acid esters of ade sine migrate in a group. To get closer formation on the composition of this terial, we analyzed the fraction for am acids after mild alkaline hydrolysis. S. Moore and H. G. Gundlach kindly hel us with this analysis, which was done the automatic Stein and Moore analy Results of three such analyses of fr liver phenol extracts are presented in t 2. The frequency pattern of the var amino acids should not be taken as meaningful because we have experien a rather different stability in this link with different amino acids. It may th fore be more a reflection of stability. any case, the experiments demonstrate

TABLE 2

no acid composition of aminoacyl adenosine fraction from soluble RNA treated with RNase

Amino acid	Amount		
	mμmoles		
Lysine	51	68	—
Histidine	14	16	—
Arginine	6	20	—
Tryptophan	0	0	0
Aspartic	24	0	34
Threonine	34	29	42
Serine	142	80	96
Glutamine	0	40	42
Proline	21	0	25
Glycine	99	54	64
Alanine	53	32	44
Cystine	0	0	0
Valine	28	58	49
Methionine	2	7	15
Isoleucine	17	14	31
Leucine	23	32	60
Tyrosine	7	9	13
Phenylalanine	2	9	16

r isolation of this fraction and release of o acids by mild alkali see Zachau *et al.* . Amino acids were determined in Stein Moore's automatic analyzer.

ellular occurrence of these amino acid rs of s-RNA. It can also be seen in e 2 that all amino acids were present pt tryptophan and cystine. Neverthe- they are known to be transferred en- ically to s-RNA and the reason for r absence from our fraction remains to xplored. It is of some importance, I that essentially all amino acid esters -RNA be freely diffusible in the cell; are the active amino acids ready to r the peptide-linking process in protein hesis.

similar question to the one already lved in the case of activating enzymes hether there is a specific RNA for every no acid. It was indicative that differ- amino acids, when used together, ved additivity rather than competition. eriments by Schweet's group (Smith *et* 59) seemed to indicate the possibility eparating specific acceptor functions. Boman has approached the question by g column electrophoresis (Porath, , with some hopeful results. A tracing ch an experiment is shown in figure 4. dotted line corresponds to the electro- esis pattern of s-RNA from calf's liver has been stripped of amino acids.

The various fractions were tested for acceptor functions, radioactive leucine and threonine being used. The two curves in figure 4 indicate that leucine–RNA moves more rapidly toward the positive than threonine–RNA. Although there is some overlap, a preliminary separation of the two appears to be fairly clear. More recent experiments by Holley and Merrill ('59), in which they used countercurrent distribution, seem to differentiate even more exhaustively between RNA's that are specific for their respective amino acids. From all this, we now feel pretty well assured that we are dealing with a "fleet" of different RNA's, each specific for only one of the whole set of amino acids.

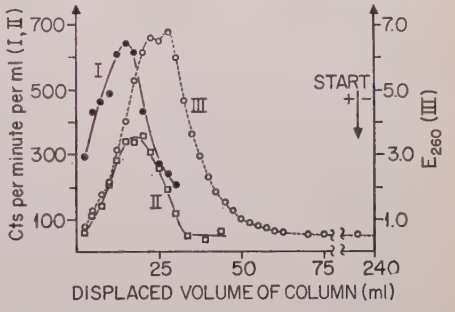


Fig. 4 Column electrophoresis (Porath, '56) of s-RNA in 3 *M* urea at *p*H 3.3. I, C^{14}-leucine uptake; II, C^{14}-threonine uptake; III, ultraviolet absorption.

During these studies, a large number of questions arose that made it very desirable to have a closer view of the mechanism of formation of this RNA–amino acid ester by transfer through the activating enzyme. It was therefore very disappointing when we failed to obtain transfer to RNA with the highly purified tryptophan-activating enzyme. This failure has now been traced to contamination with RNase. But at that time, we really began to worry about whether the activation and transfer reactions in the animal might not be split up into two independent enzymic steps. Therefore, Dr. Hartmann tried to find another suitable enzyme of animal origin for purification. Calf's liver was found to be a good source, and the threonine-specific enzyme was chosen because it was rather active and could be fairly easily concentrated, as shown in table 3. Most important of all, the transfer to RNA

TABLE 3

Threonine specificity of 250-fold purified enzyme

Amino acid	Specific activity	Percentage[a]
	units/mg of protein	
Threonine	690	100
Phenylalanine	51	7.4
Tryptophan	35	5.1
Isoleucine	2.8	0.4
Serine	2.7	0.4
Valine	2.7	0.4
Leucine	1.7	0.2
Alanine	1.7	0.2

[a] Amino acid-dependent PP^{32}-ATP exchange was measured.

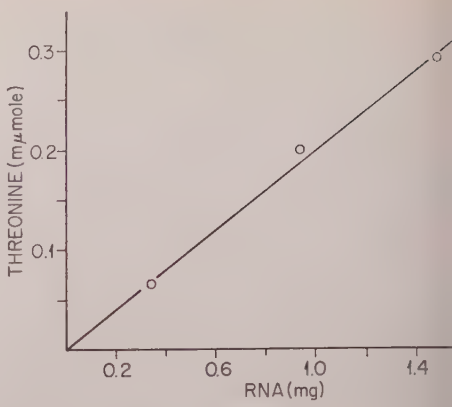


Fig. 5 Threonine transfer to RNA. For cedure, see Assay II, table 4.

could easily be followed with this enzyme. Figure 5 shows proportionality with added RNA at lower RNA concentrations. Therefore, the enzyme can be used for assay of threonine-specific RNA. On the other hand, when it is saturated with RNA, transfer of threonine to RNA may be used to assay this function as well as for comparison with the ATP-pyrophosphate exchange assay, which is specific for the first step and independent of the presence of s-RNA.

When these two assays are used, it appears from table 4 that, at various degrees of purification, their ratio remains quite constant. We conclude from these results that activation and transfer in the animal system is a function of the same enzyme, analogous to that established for microbial systems (Berg, '58). With this enzyme confirmed the over-all reversibility of

$$\text{ATP} + \text{threonine} + \text{RNA} \rightleftharpoons \text{AMP} + \text{RNA threonine ester} + \text{PP}$$

As shown in table 5, addition of both A and PP is necessary to revert from amino acid ester to ATP. The equilibr constant of this reaction was found t 0.7, which indicates that ATP and amino acid ester are on a rather sim energy level. There are probably two sons for this high group potential in "ordinary" ester: (1) an amino acid e may be strained through the amino g neighboring the ester bond; (2) the jacent free hydroxyl may have a sim effect. In this connection, one remem

TABLE 4

Comparison of activation and transfer to s-RNA with various preparations of threonine-activating enzyme

Enzyme preparation	I mμmoles of PP^{32} exchanged per mg of protein	II mμmoles of threonine transferred to RNA per mg of protein in 10 min	III Ratio
1. Dialyzed 100,000 × *g* supernatant of homogenate	1.0	—	—
2. 0.55–0.85 $(NH_4)_2SO_4$-cut	7.8	87	0.10
3. First separation on DEAE-cellulose column	43.6	345	0.13
4. Second separation on DEAE-cellulose column	138.0	1285	0.11

Assay for I: 3 μmoles of ATP, 3 μmoles of PP^{32}, 4 μmoles of $MgCl_2$, 9 μmoles of KF, 0.7 μmole of L-threonine, 100 μmoles of Tris-HCl *p*H 7.7, enzyme. Total volume 1.0 ml, 10 minutes' incubation at 37°C. After deproteinization with TCA, the exchange was determined as described by Davie *et al.* ('56); ATP^{32} was counted on the washed charcoal.

Assay for II: 2.35 mg of RNA (saturating), 3.75 μmoles of ATP, 10 μmoles of $MgCl_2$, 0.1 μmole of C^{14}-L-threonine, 100 μmoles of Tris maleate *p*H 6.7, enzyme units as given. Total volume 1.0 ml, 10 minutes' incubation at 37°C. After incubation, the charged RNA was precipitated with 8 ml of acetic acid alcohol (Weiss *et al.*, '58), washed three times with 5% TCA and once with ethanol.

TABLE 5

Reversibility of threonine incorporation into RNA

Reaction mixture			Threonine incorporated into RNA
			mμmole
5 minutes' incubation			0.24
15 minutes' incubation			0.26
After 5 min	+ 1.8 μmoles of AMP + 2 μmoles of PP	and 10 min more	0.04
	+ 1.8 μmoles of AMP		0.22
	+ 2 μmoles of PP		0.21

Procedure similar to Assay II, table 4.

reluctance with which the energy-rich ure of the thioester bond was originally epted, since there had been a tendency iew the oxygen ester and the thioester equivalent structures.

PROTEIN SYNTHESIS

he chemistry of the final stage, when vated amino acids line up into an erly sequence, is still fragmentary. at we know about the microsomal sys- has been largely worked out by the ecnik group (Hoagland *et al.*, '58). h the isolated threonine-activating en- e, we felt that we were in a good posi- to explore a question so far unan- red: if a single activated amino acid be accepted by the microsome or if, hown in many cases of protein synthe- the total complement of amino acids is essary. A representative experiment in ch our threonine-activating enzyme used is reproduced in table 6. The ll incorporation in number 1 may be inal (Webster, '59), but we did not mpt to go into this. However, addition ther amino acids and their enzymes 2) causes a fourfold increase in label- This result shows that, in the iso- d liver system, the complete set of no acids appears to be required for in- oration into protein.

Following further the path of the activated amino acid in its formation of a protein, we will briefly mention some experiments in this area that Drs. Acs and Hülsmann have done recently. Dr. Hülsmann confirmed the observation by Keller and Zamecnik ('56) of a need for a rather large supply of ATP and catalytic amounts of GTP for this terminal transfer into protein to occur. The manner in which these two factors participate is rather puzzling if we think of the amino acids in ester link to RNA as being the immediate precursors of sequential peptide links in the protein. The relatively large amount of ATP, of course, makes further energy turnover in this step a possibility, particularly with the additional appearance of GTP as an ingredient in the final step. As long as this mystery is not resolved, there is very little chance of reasoning in chemical terms about the mechanism of this step. A certain impression of the reaction is given in the experiment represented in table 7, which shows that the absolute amount of amino acid transfer is independent of the amount of added amino acid-carrying RNA but that it is rather clearly dependent on the amount of microsomes. When carried out at 25°C., the reaction was linear during the first 5 minutes. If a low concentration of active

TABLE 6

Comparison of threonine transfer from RNA to microsomes, with or without other amino acids

Number		Radioactivity
		cts/min
1.	RNA fraction, labeled with threonine only	100
2.	RNA fraction, labeled with threonine + other amino acids	425

No. 1—Stripped RNA, charged by purified threonine-activating enzyme with 1000 cts/min C^{14}-threonine.

No. 2—Stripped RNA, charged by crude supernatant, with 1000 cts/min C^{14}-threonine and all other amino acids.

TABLE 7

Transfer to microsomes from varying amounts of C^{14}-leucine-RNA

Counts added	Counts transferred after	
	2½ min	5 min
cts/min	*cts/min*	*cts/min*
2560	89(3.4%)	163(6.4%)
1280	86(6.7%)	140(10.9%)
640	64(10.0%)	124(19.4%)
320[a]	59(21.6%)	120(37.5%)

[a] 6.4×10^{-8} *M* leucine-RNA. 5.7 mg (dry weight) of microsomes in 1 ml at 25°C.

amino acids was used, 40% of it found its way into the protein. Occasionally, nearly all of the amino acid was transferred. The high degree of incorporation indicates high efficiency of the transfer under most-favorable conditions, and rather little side reaction of the active amino acid.

For whatever they may be worth, I still want to mention briefly experiments by Dr. Hülsmann on the temperature coefficient of transfer to microsomes. As shown in table 8, there is a break in the temperature curve between 20° and 17.5°C. Interpretation of such a finding,

TABLE 8

Temperature effect on transfer from RNA to microsomes

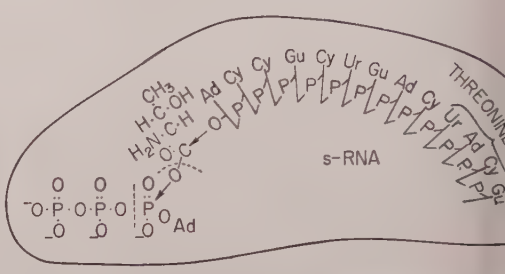
Temperature	Counts transferred
°C.	*cts/min.*
35	388
30	250
25	172
20	101
17.5	24
15	12

A total of 810 cts/min of C^{14}-leucine-RNA was added in each sample and 5 mg (dry weight) of microsomes was used as acceptor. Incubation time, 5 minutes.

it seems to me, is rather hazardous. We might refer, however, to work by Koshland's group (Levy *et al.*, '59) on a similar break in the temperature curve with the myosin-ATP reaction, which they relate to a structural change in the protein.

CONCLUSION AND OUTLOOK

In reviewing the whole reaction sequence, we find that amino acid-activating enzymes may be considered as reactors that bring together an amino acid, and only one particular amino acid, with specific RNA, which, in its nucleotide rangement, must contain a configurat that specifically attaches to this particu enzyme. We might express this somewh fancifully by saying that the enzyme actor translates amino acid specificity i RNA specificity. In figure 6 we try



ATP + THREONINE + RNA ⇌ THREONINE ~ RNA + AM

Fig. 6 Amino acid activation enzyme.

describe how, with ATP as the ene source and liberating inorganic PP in initial step, the energized aminoacyl c tacts the terminal adenosine of RNA the enzyme. Its terminal cytidylic-cyti lic-adenylic sequence appears to be co mon in all s-RNA's irrespective of amino acid attached, and on this end, RNA behaves as a relatively nonspec cofactor. The amino acid-charged R then appears to leave the activating zyme and enter a cellular pool.

Following its paths further, and leani on Crick's adaptor theory ('57, '58), are tempted to propose base pairing a means of linking the amino acid-spec part of the s-RNA to a corresponding

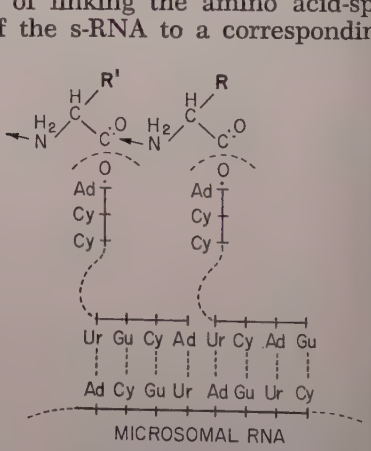


Fig. 7 Sequential peptide synthesis on t plate.

nce on the microsomal RNA. This es us at least one way of visualizing nation of the sequence on the template assuming hydrogen binding of uridylic denylic, and guanylic to cytidylic acid. s incorporated in figure 7 and should be en for not more than a very rough at- pt to make some sense of a chemistry t could operate in the final sequential- ion step.

OPEN DISCUSSION

QUESTION: Dr. Lipmann, is it always adenosine that is esterfied by the amino d?

LIPMANN: So far, we can say that it is bably always the adenosine, which fits o with the terminal in soluble RNA, ng uniformly cytidylic-cytidylic-adeny- acid.

CRICK[2]: If there had been another ter- nal one, could you have picked it up?

LIPMANN: I think so. If there were one ink we should have picked it up but not races, however.

CRICK: You have picked it up for about of them or something like that?

LOFTFIELD[3]: I should like to mention t 18 of the amino acids specifically re- red the ACC terminal group before ng attached to soluble RNA; I think that s is pertinent to the question.

CRICK: Yes, that is true, but the ounts, if I recall, were quite variable d they were not convincing for some of amino acids.

BERNHARD[4]: On the basis of what you ve said, Dr. Lipmann, do you believe t all or essentially all of the specificity the selection of amino acid lies in one the first two steps, that is, in the steps uiring activating enzyme?

LIPMANN: It is a little difficult for me answer this, because the term "specific- " is somewhat flexible. We can only say present that there is apparently for ery amino acid an activating enzyme d a particular RNA. Dr. Crick, do you ve some comment on the specificity?

CRICK: I would like to ask the same estion of Dr. Loftfield. Would you like comment on this question as to how ch of the specificity generally lies in the t two steps?

LOFTFIELD: I think that we do have a pertinent experiment. Dr. Lisa Hecht and I prepared a compound L-*allo*isoleucine-C^{14}, which is very closely related to both valine and isoleucine, and we demonstrated that it gets into the cell as well as the others. It is discriminated against in the labeling of the RNA approximately 20 times to 1; i.e., it goes on to RNA about one-twentieth as well as isoleucine does. This seems to be a quite ordinary competitive problem; 20 times as much isoleucine as *allo*isoleucine gives a 50% inhibition. In the over-all conversion into protein, however, the *allo*isoleucine is discriminated against at a ratio of something like 1: 2000, depending on the tissue. From this we would be inclined to guess that there may be two stages at which discrimination operates. Certainly, though, there is a great deal of discrimination in the first step.

NOVELLI[5]: Dr. Lipmann, what do you think about M. Beljanski and S. Ochoa's experiment in which the *p*H 5.0 fraction of liver supernatant is replaced by the incorporating enzyme from *Alkaligenes faecalis*? The incorporating enzyme is apparently devoid of activating enzymes that make the aminoacyl adenylates; yet, when this enzyme is mixed with rat liver microsomes, the same amount of incorporation of amino acid is observed as when the *pH* 5.0 fraction of rat liver supernatant is used.

I should also like to know what relation Dr. Fruton's very interesting experiment, in which he found that in a mitochondrial incorporating system all of the incorporated amino acid is N-terminal, may have to the microsomal incorporating system.

LIPMANN: The Beljanski-Ochoa experiments could indicate an entirely different system. It seems unlikely that there could be enough enzyme plus RNA in the microsome to carry on some of the steps that we have been talking about here. Then, to assume an alternative activation system would be the only explanation, which I am

[2] F. H. C. Crick, University of Cambridge.
[3] R. B. Loftfield, Massachusetts General Hospital.
[4] Sidney Bernhard, National Institutes of Health, Bethesda.
[5] G. D. Novelli, Oak Ridge National Laboratory.

not too happy with. We have just decided that we find a good amino acid specificity in this preliminary reaction. We also have experiments showing that, if we leave out RNA, the transfer does not work. I am unable to explain this paradoxical situation.

FRUTON[6]: I think that it is too early to decide whether the experiments that Dr. C. Zioudrou and I reported—about the labeling of mitochondrial protein by amino acid adenylates or by labeled amino acid residues transferred to the mitochondrial protein by the catalytic action of proteolytic enzymes—have direct bearing on the question of protein synthesis. The experiments do, however, indicate the need for caution against over-ready interpretation of incorporation studies as a measure of protein synthesis; and I think that this perhaps is the point Dr. Novelli wanted to bring out.

May I, however, take this opportunity to mention the much more pertinent work of my colleague Dr. M. V. Simpson, who is studying the synthesis of cytochrome c by the mitochondrial fraction of beef heart muscle. He has shown that a fortunate property of this preparation enables it to continue to incorporate labeled amino acids as long as 16 hours, in contrast to the 20 minutes or so for the comparable liver preparation. With the muscle mitochondria, we can demonstrate a net increase in the amount of cytochrome c that can be isolated. It is clear that here we are dealing with an increase in the amount of labeled protein and, what is more important, working on the biosynthesis of a protein that can be isolated in as homogeneous a form as one can isolate a protein today. Dr. Simpson has obtained the cytochrome c preparation in a form that conforms with the properties of cytochrome c as isolated by the various investigators who have worked on it for many years.

From this cytochrome c Dr. Simpson obtained the heme peptide by peptic degradation as described by H. Tuppy and S. Paleus and has demonstrated that, if C^{14}-valine is used as the labeled amino acid, labeled valine appears in its appointed place in the peptide chain of the heme peptide. You may remember that this heme peptide contains 11 amino acids, two which are valine residues. And may just add for the consideration of anyo who wishes to make a hypothesis abo the mechanism of protein synthesis th if the two valines are separated from ea other—this is possible by virtue of the f that there is a lysine residue between the thus permitting tryptic cleavage of t heme peptide at the lysine carbonyl gro —and their respective specific radioactiv is measured, the ratio of the specific rad activities is roughly 11:1. This is about much as I think I should say about Simpson's experiments. Some of this currently in press. There is more wo and I hope he will report on it at t Atlantic City meetings. [Ed. note: Fede tion Proc., *18:* 187, 1959.]

LIPMANN: I have greatly admired Simpson's work, and when he repor recently at the Rockefeller Institute we were quite impressed by it. I was in a w a little pleased when, in the discussi afterwards, Dr. Simpson said that he h originally thought that he was deali with a quite different system from the o that was studied with the microsor But, since he has now recently fou amino acid-activating enzymes in the m tochondria, he is at least open to the s gestion that the interpretation of the p tein synthesis in the mitochondria mi go by a pathway similar to the one in microsomal system.

CRICK: The simple hypothesis to plain Simpson's results is that a he peptide is made separately from the m body of the protein. I think Dr. Lipma has been talking about the initial steps protein synthesis, whereas Dr. Simpson studying perhaps one of the final ste Of course, the most interesting steps, has been said, are in the middle. We h not got to them yet.

LYNEN[7]: It is interesting that you f 40% of the linked RNA is bound to ser and glycine. I also note that it is diffic to separate the various soluble RN Since you have purified the threonine tivating system, have you considered

[6] J. S. Fruton, Yale University.

[7] Feodor Lynen, Max-Planck-Institut für chemie, München.

sibility of isolating a specific RNA as threonine complex? The altered rge of the complex would facilitate its aration from the free RNA's.

LIPMANN: Maybe Dr. Boman can comnt on this. He has been working on s question.

BOMAN: Well, that is how I actually rted to do it, but it was much easier say than do. The problem is that the t of the electrogram where the charged A should appear is covered by RNA t we have called "junk RNA" and must nove in order to get that spot free. We ve also experiments indicating that, if start with labeled RNA, the label will ve slightly slower than the bulk of the A.

CRICK: Isn't this possibly RNA that is t taken up by the amino acid?

BOMAN: It could be.

CRICK: But there must be the other A's there. Actually, I have views on question: how do you fractionate the uble RNA to obtain a fraction that will e up only one amino acid? I think it is ely to be difficult by conventional meth-. Perhaps the best way would be to el the soluble RNA with one amino d and use this as a protective group.

BOMAN: I want to take out the active ction and label it with a purified amino d-activating enzyme and then rerun it the hope that the amino acid-RNA will pear as a small peak behind the other A.

BRENNER[8]: Dr. Lipmann, what makes ino acid esters of 1,2 glycols apparently re reactive than amino acid esters of nohydroxy alcohols?

LIPMANN: Is Dr. Khorana here?

KHORANA[9]: Two points may be made. st, I think that it is correct to say that α-amino acid esters are more reactive bile) than the esters of simple carylic acids.

Second, we also know chemically that if measure of rate of hydrolysis is any ication, then in the ribonucleoside ser, the 2′ or 3′ acetates are far more labile n the nucleoside 5′ monoacetates to aline hydrolysis. Organic chemists have ed likewise the catalytic action of the ghboring hydroxyl function on hydrolyof esters. I think the presence of the hydroxylic function is certainly a contributive factor, as well as the fact that the aminoacyl radical is a rather different radical.

CRICK: Why should that affect the equilibrium rather than the rate?

KHORANA: It is more labile.

CRICK: Yes, but that is really a rate.

KHORANA: Yes, it is more labile and this should be a measure of its reactivity or high-energy character of the bond.

LIPMANN: It is true that we talk about a rate, but reactivity and thermodynamic potential sometimes, although not always, seem to run parallel. As shown in figure 1 of our paper, reactivity with hydroxylamine runs rather parallel with thermodynamic potential. It still is only an approximation, I am sure.

BRENNER: I might add something. Dr. Crick said that the second hydroxyl group in the glycol might affect the rate of hydrolysis and he raised the question of how it influenced the equilibrium. An equilibrium may be considered as being determined by the ratio of the forward and backward reactions and so an increase in the rate of the forward reaction might be a likely explanation.

Actually, about 10 years ago, we worked a bit with amino acid esters and, among other esters, we prepared those of methylcellosolve and ethylene glycol. There is an extreme difference between these two. Insofar as the esters of glycol are concerned you can easily see how they are converted into crystalline diketopiperazines. As a matter of fact, diketopiperazine formation was much faster with the glycol than with the methyl ester. So there is certainly an influence on reaction rate and, apart from this, of course, you cannot compare an amino acid ester with ethyl acetate. In my opinion, therefore, Dr. Lipmann's finding that a compound analogous to an amino acid glycol ester is energy rich is not so very astonishing.

AUGENSTINE[10]: Part of your material is rather disappointing from a "coding" as-

[8] Max Brenner, University of Basel.

[9] H. G. Khorana, British Columbia Research Council, University of British Columbia.

[10] L. G. Augenstine, U. S. Atomic Energy Commission, Washington.

pect. In particular, the occurrence of an identical trinucleotide terminal grouping on the different soluble RNA's reduces the specificity that could be obtained from the attachment of an amino acid to nucleotides. Therefore, do you have any evidence that there is an intimate reaction or complex between the activating enzymes and the soluble RNA's? For instance, do you find that the length of the RNA is correlated with the molecular weight of the corresponding activating enzyme?

Also is there any evidence that amino acids such as serine and glycine (which can be the "heads of amino acid families") are first bound in the normal form to the soluble RNA but are then changed on the microsomes into a different member of that amino acid family? Experimentally, this would cause the concentration of a particular amino acid bound to the soluble RNA to be different from that occurring on the microsome.

LIPMANN: I should like to point out that in the tables in our paper the amino acid carrying RNA was isolated from rat livers and treated rather carefully. But still this is a kind of hit-or-miss thing, and I do not know whether the abundance of one amino acid against another is attributable to a difference in stability. I am inclined to believe that, at least in part, it may be so. I do prefer to consider these data as not representative of actual abundance of various bound amino acids.

AUGENSTINE: Since serine and glycine are "heads of families" in some organisms, the high levels of activation that you reported made me suspect that this might be a possibility. To my knowledge, Dean B. Cowie at the Carnegie Institution of Washington was the first to suggest that amino acids might be bound to soluble RNA in one form and then changed at the microsome site.

CRICK: I do not think it is likely, but I should like to ask this question: Is there a case of two amino acids that are members of the same family for which the activating enzymes have been found?

LIPMANN: I know that the serine enzyme is now being worked on.

NOVELLLI: No glycine yet that I know of.

LIPMANN: L. T. Webster and E. Davie ('59) have an abstract on serine-activating enzyme in the Federat Proceedings.

STROMINGER[11]: Perhaps Dr. Lipma would like to comment on the paper Schweet *et al.* ('58) on the synthesis hemoglobin. In synthesizing hemoglob they used *p*H 5 enzymes with soluble R from guinea pig livers and the microsor from rabbit reticulocytes. Presumably, t means that the specificity of the solu RNA has very little to do with the cod mechanism, at least in the synthesis of t one protein.

My question perhaps is related. C of the unexpected things about the tra fer reaction to RNA is its reversibility. there really good evidence that the ami acyl-RNA transfers amino acid directly the microsomal system or might it not back through the aminoacyl adenylate fore transfer to the microsomal system

LIPMANN: This seems to be exclu because we do not need the activating zyme for the transfer of the amino a from RNA to the microsome. As to question whether the coding is in soluble RNA, I would agree that solu RNA has nothing to do with the codi The coding is on the microsome. Actua the supernatant system is relatively n specific. Dr. J. Mager found that *Tetrahymena* supernatant can substit in the liver system and liver supernat can substitute with the *Tetrahymena* crosome. So the supernatant, I think, though it carries the specificity for amino acid, does not really have any lation to the coding.

CRICK: I would use the word differen I use the word coding to mean short quences of bases that correspond to e particular amino acid, and I believe t such a sequence does exist on the solu RNA. If I may answer Dr. Stroming question (as to whether we would exp the code to be universal), we would exp that there would be the same seque on the soluble RNA from any species; slightly embarrassing that there is s unpublished work that makes this likely.

[11] Jack Strominger, Washington University Louis.

STROMINGER: The code obviously is not ite fixed for RNA that can accept methi- ine because there are at least two dif- rent RNA's that can accept methionine rom Paul Berg's experiments with yeast d microbial methionine-activating en- mes).

LIPMANN: I have to take back my for- er statement because I have to agree with . Crick that soluble RNA's carry the hers which may indeed be universal. was focusing on the assembling of the hers into specific units when I was lking about the microsomes; but both e part of the coding system.

CRICK: That could be because there is me coenzyme that is not quite the same. vould just like to report that Dr. Geoffrey own (personal communication) has own that the reason the tyrosine enzyme es not transfer is that it is contaminated nucleases and if you remove these you n get transfer of tyrosine from the ac- ating enzyme to the soluble RNA.

MCCORQUODALE[12]: Dr. Lipmann, is the nsfer of the aminoacyl group from the inoacyl-s-RNA to the microsomes an en- mic reaction, and if so, in what cellular action is this enzyme found?

LIPMANN: This is a crucial question. e have tried to find the enzymes for is transfer step from the soluble RNA the microsome, and P. Zamecnik's oup I am sure is working hard on it.

MCCORQUODALE: Do you know of a re- irement for the presence of a soluble action for the transfer?

LIPMANN: We are not sure about this. e would like to assume it, but we have shed the microsomes; if we add mag- sium, then they become rather stable d a good deal of protein could be washed . Dr. Hülsmann has done such experi- nts, but so far we have not been able resolve the system.

[12] D. J. McCorquodale, Emory University.

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dol and Ketol Condensations

B. L. HORECKER
New York University College of Medicine, New York City

erhaps the two most common biolog- mechanisms for extending and short- ng carbon chains are the aldol and ketol densations. These may be represented shown in figure 1.

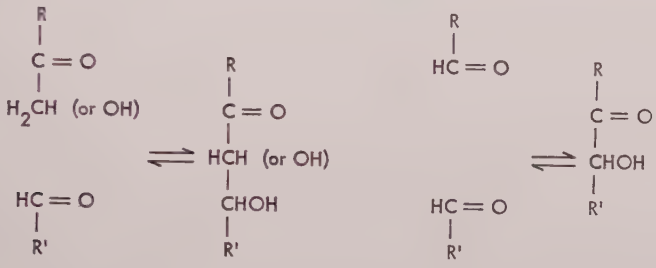

Figure 1

he aldol condensation involves a car- -bound hydrogen adjacent to a carbonyl up and yields a structure containing the roxyl group β to the carbonyl function. the ketol condensation two carbonyl npounds react and the product contains hydroxyl group adjacent to the car- yl function.

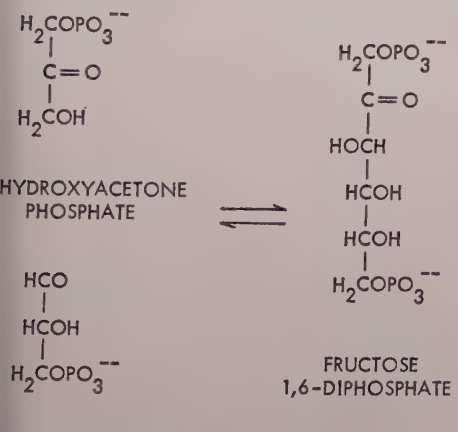

Figure 2

A number of different types of aldol and ketol condensations are known, and these have now been implicated in fatty acid and amino acid metabolism as well as in carbohydrate metabolism. Some of these reactions will be discussed in other papers. Obviously, all of them cannot be considered or even briefly reviewed in a limited space; I shall therefore confine my remarks to a few selected cases for which some basis exists for a discussion of the enzymic mechanisms.

Fructose diphosphate aldolase. The reversible cleavage of fructose diphosphate to form 2 moles of triose phosphate is one of the oldest known and most thoroughly studied reactions of the aldol condensation type (fig. 2). The enzymic reaction was discovered by Meyerhof and Lohmann ('34), although the alkali-catalyzed condensation of the free trioses had been described many years earlier by Fischer and Tafel (1887).

The enzymic reaction is completely specific for dihydroxyacetone phosphate, whereas the other reactant, glyceraldehyde 3-phosphate, can be replaced by any of a large number of aldehydes including erythrose 4-phosphate (Horecker *et al.*, '53), glycolaldehyde phosphate (Byrne and Lardy, '54), and a number of nonphosphorylated aldehydes (Meyerhof *et al.*, '36a, b).

With respect to the mechanism of activation of dihydroxyacetone phosphate, some interesting experiments have been reported by Rose and Topper and their coworkers (Topper *et al.*, '57; Rose and Rieder, '55, '58; Bloom and Topper, '56, '58; Rose, '58a) as well as by Rutter and Ling ('58). The important observations are (1) exchange of one atom of carbon-bound hydrogen with the aqueous solvent, (2) the steric specificity of this exchange, and (3) the formation of an ultraviolet-absorbing compound, presumably the enediol or carbanion resonance form, which appears when the enzyme is mixed with dihydroxyacetone phosphate. These observations led to the suggestion that an enzyme-bound carbanion was formed that was in equilibrium with the enzyme-bound enol form (fig. 3). Stereospecificity of dihydroxyacetone phosphate activation by the enzyme was deduced from the fact that only one atom of tritium was incorporated when the substrate was incubated with the enzyme in T_2O. This conclusion was confirmed when it was established that the exchange reactions catalyzed by aldolase or triosephosphate isomerase involve different hydrogen atoms. Thus, when dihydroxyacetone phosphate labeled by incubation with aldolase is reincubated in ordinary water with triosephosphate isomerase, no tritium is lost. Conversely, if label is introduced by incubation in T_2O with triosephosphate isomerase, then it is not removed by incubation in H_2O with aldolase.

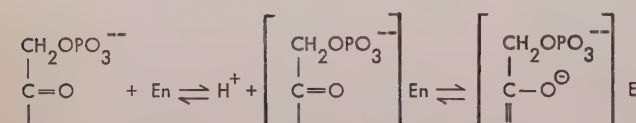

Figure 3

The exchange experiments indicate that the formation of free dihydroxyacetone phosphate from fructose 1,6-diphosphate requires at least three steps: (1) cleavage of fructose 1,6-diphosphate to yield glyceraldehyde 3-phosphate and the carbanion form of dihydroxyacetone, (2) uptake of a proton, and (3) dissociation of dihydroxyacetone phosphate from the enzyme complex. On this basis we might expect that exchange of glyceraldehyde 3-phosphate with fructose 1,6-diphosphate wo be more rapid than exchange of dihydr acetone phosphate. This has been dem strated by Rose ('58b), who found exchange of labeled fructose diphosph with glyceraldehyde 3-phosphate to be times as rapid as exchange with dihydr acetone phosphate.

This important observation may h to explain a number of previous findi relative to the formation of asymmetric labeled hexose from C_3 precursors (Lo *et al.*, '50; Dische and Rittenberg, Marks and Horecker, '56). In these exp ments three-carbon compounds, precur of lactate or pyruvate, introduced m label into the lower half of the hex molecule. This was usually attributed lack of equilibration of the two triose p phates, resulting in a relatively cold d droxyacetone pool; it may, Rose points out, be caused part by a more rapid excha of glyceraldehyde 3-phosph with endogenous fructose phosphate. It would be of in est to check the implication this experiment by direct m urement of the Michaelis-Menten affi constants for the two triose phosphates though in this case, rate of dissocia from the enzyme may be more impor than equilibrium constant. However, explanation does not account for the as metry obtained by Schambye *et al.* (with glycerol as the hexose precursor. this case the top half of the molecule more heavily labeled, and lack of comp equilibration by triosephosphate isome remains the most reasonable explanat

Direct spectrophotometric evidence an enzyme-bound enol was obtained Topper *et al.* ('57). A mixture of d droxyacetone phosphate and stoichiome amounts of muscle aldolase absorbs in ultraviolet region below 250 mμ, as w be expected for the enolate structure. absorption is not obtained with glycera hyde 3-phosphate in place of dihydroxy tone phosphate.

Bloom and Topper ('58) deduced absolute configuration of the dihydr acetone carbanion from the nature of condensation product formed with gly aldehyde 3-phosphate. Rose ('58a)

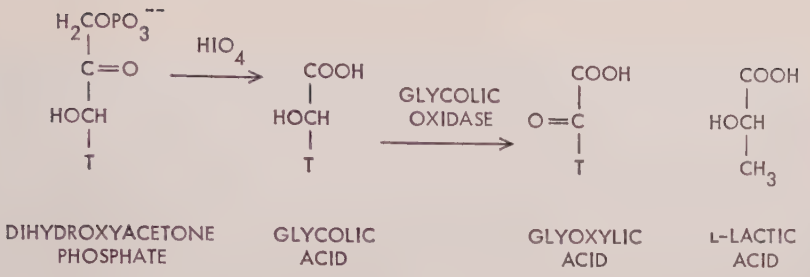

Figure 4

ned their conclusion in an elegantly de-
ned series of experiments. Dihydroxy-
tone phosphate, labeled with tritium by
ubation in T_2O in the presence of aldo-
e, was dephosphorylated and oxidized
h periodate to yield tritium-labeled
colic acid (fig. 4). Glycolic acid
s, in turn, oxidized enzymically to
oxylic acid with glycolic oxidase with-
loss of tritium. This enzyme will
dize L-lactic acid but not the D
m. It was concluded that the glycolic
d formed, and therefore the tritiated di-
droxyacetone phosphate, carried the
el in the same relative position occupied
the proton in D-lactic acid. By contrast,
en the experiment was performed with
ydroxyacetone phosphate labeled by in-
bation in T_2O with triosephosphate iso-
rase, the opposite result was obtained.
this case tritium was completely re-
ved by glycolic oxidase; it must there-
e have occupied the same position as the
roton in L-lactic acid.

On the basis of the observations sum-
rized above, Bloom and Topper ('58) at-
tributed aldolase specificity to two factors:
(1) three-point attachment of dihydroxy-
acetone phosphate to the enzyme to yield a
stereospecific carbanion. As a result, only
two of the four possible condensation pro-
ducts can be formed; namely, those pos-
sessing the L configuration on C-3, fruc-
tose, or tagatose (fig. 5). However,
(2) although tagatose diphosphate is
slowly split by aldolase (Tung *et al.*,
'54), it is not formed in the condensa-
tion, which yields only the fructose ester.
This second factor may not be enzymic,
since it operates as well in the alkali-cata-
lyzed condensation, where only the *trans*
isomers are formed. It might be consid-
ered, therefore, that the sole activating
function of the enzyme relates to the di-
hydroxyacetone phosphate moiety. This
seems unlikely, however, in view of the
great difference in reactivity between such
substrates as glyceraldehyde 3-phosphate
and D-erythrose 4-phosphate, on the one
hand, and their nonphosphorylated coun-
terparts on the other hand. This prefer-
ence for the phosphorylated aldehyde de-

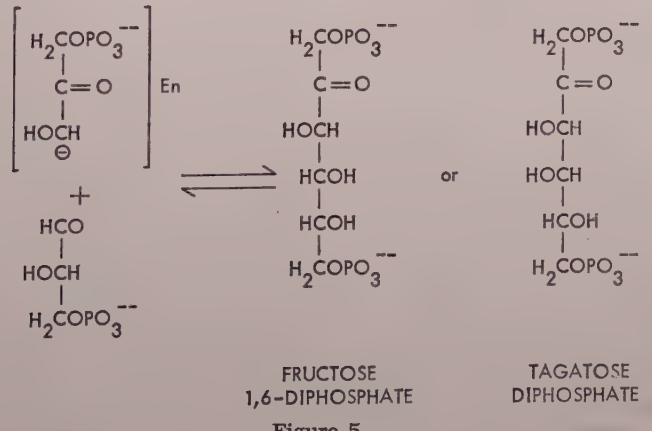

Figure 5

rivatives is an indication that binding of the aldehyde moiety by the enzyme is important, if only to bring this reactant into favorable proximity and to permit it to compete effectively with protons from the medium. The effect of phosphate is even more dramatic with the C_5 sugars. Dische ('58) reported octulose diphosphate formation with ribose phosphate, but the free pentoses are not known to react.

Although not strictly relevant to the mechanism of the reaction, a few comments regarding the equilibrium may be of interest. Meyerhof *et al.* ('36b) reported the formation of fructose 1-phosphate from dihydroxyacetone phosphate and D-glyceraldehyde to be an irreversible reaction, but Tung *et al.* ('54) later demonstrated some cleavage of the product. Lehninger *et al.* ('55) showed that the stability of the condensation product is correlated with the ability to form the pyranose ring form. Products such as fructose 1-phosphate, which can form the pyranose ring, were found to be most stable, whereas those unable to cyclize, such as 5,6-dideoxyfructose 1-phosphate, were cleaved to the greatest extent. Those able to form the furanose ring were of intermediate stability.

Deoxyribosaldolase. Several years ago Racker ('52) discovered an enzyme that catalyzes the reversible splitting of deoxyribose 5-phosphate (fig. 6). We encountered it again as an inducible enzyme in the pathway of deoxyribose fermentation by *Lactobacillus plantarum* (Domagk and Horecker, '58). Pricer has now purified the enzyme from extracts of this organism, and a few of his observations may be pertinent in connection with the mechanism of action of aldolases. The

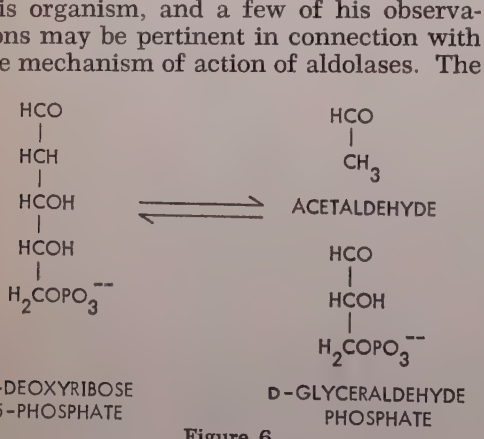

Figure 6

reaction is readily reversible, with an eq librium constant at 37°C. of 2×10^{-4} When the reaction is studied in the dir tion of condensation, the affinity for ac aldehyde is found to be only slightly l than that for glyceraldehyde 3-phosph (table 1). This is in contrast to Racke report for the enzyme isolated from *cherichia coli* and a synthesis of deo ribose 5-phosphate from acetaldehyde n not be entirely excluded on substrate finity grounds.

TABLE 1

Affinity constants for deoxyribosaldolase

Substrate	K_s
Glyceraldehyde 3-phosphate	7.1×1
Acetaldehyde	1.1×1
Deoxyribose 5-phosphate	6.4×1

Some information is now available the mechanism of this reaction. The zyme appears to be specific for aceta hyde; i.e., this reactant is not replaced glycolaldehyde or propionaldehyde. It be of interest to repeat the tritium change experiments with the deoxyri aldolase system to determine whether banion formation occurs, but in any c a stereospecific exchange is impossi since the carbon atom carries three hy gen atoms rather than two, unlike di droxyacetone phosphate. Because, th fore, activation of acetaldehyde can affect the configuration of the ne formed C—C bond, other factors must termine the nature of the final prod

Since theoretically two compounds be formed in such a condensation it important to eliminate 2-deoxy-D-xylos a possible product (fig. 7). Pricer m ured the optical rotation of the product concluded that it contained only the ribose isomer. Thus, in this case, product with the *cis* configuration al is formed.

A number of compounds will rep glyceraldehyde 3-phosphate in the r tion (table 2). These include D-eryth 4-phosphate, which is only about a h dredth as active as D-glyceraldehyde phosphate, and the diose and pentose p phate esters, which are still less act Small but definite activity is obtained

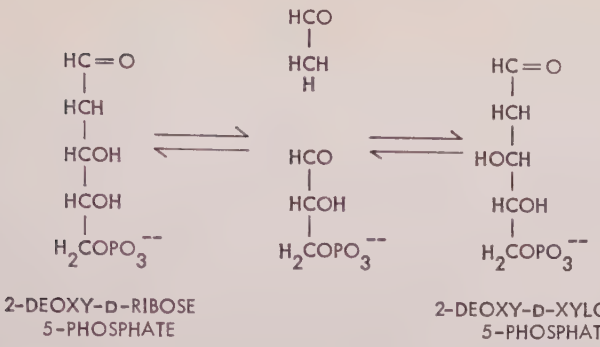

Figure 7

e triose and tetrose but not with ribose glycolaldehyde.

Of particular significance for an understanding of the mechanism of the reaction the condensation of acetaldehyde with

TABLE 2

Substrate specificity of deoxyriboaldolase

Substrate	Relative activity
Glyceraldehyde 3-phosphate	4190
rythrose 4-phosphate	40
colaldehyde phosphate	1.4
bose 5-phosphate	1.3
Glyceraldehyde	3.8
rythrose	2.4
bose	0
colaldehyde	0

lyceraldehyde 3-phosphate (fig. 8). In eriments with the D,L derivative, we nd that the total deoxy sugar produced s equal to twice the D-glyceraldehyde 3- sphate added, and after reduction of D form with DPNH and glycerophos- te dehydrogenase, the amount of de- sugar formed was reduced to exactly -half. The L form reacts about one- h as rapidly as the D form, but with icient enzyme the reaction proceeds oothly to completion. The product of reaction with L-glyceraldehyde 3-phos- te has now been identified by paper omatography as the phosphate ester of eoxyxylose rather than the 2-deoxy-L- se (fig. 9). This permits us to speculate ut the nature of the condensation cata- d by deoxyriboaldolase. If the adjacent roxyl, through internal hydrogen bond nation, were important in determining configuration of the final product, then would expect 2-deoxy-D-ribose phos- te from D-glyceraldehyde 3-phosphate and 2-deoxy-L-ribose phosphate from L-glyceraldehyde 3-phosphate. The fact that the product always shows the same configuration about C-3, regardless of the configuration of the adjacent hydroxyl, suggests that the enzyme induces a sterospecific polarization of the carbonyl group.

It will be of interest to determine as well the configuration of the products formed with the tetrose and diose esters. By analogy with the condensation shown, the hexose formed with D-erythrose 4-phosphate should be 2-deoxy-D-allose 6-phosphate.

Condensations involving pyruvate and phosphoenolpyruvate. The first reactions of this type were discovered by Doudoroff

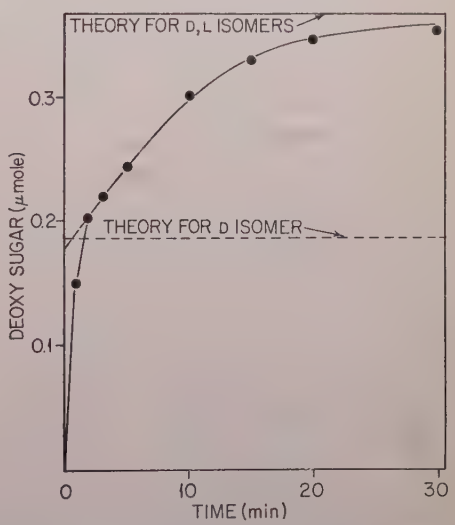

Fig. 8 The reaction of acetaldehyde with D,L-glyceraldehyde 3-phosphate, catalyzed by deoxyribosaldolase from *L. plantarum*. The reaction mixture contained an excess of acetaldehyde and deoxyriboaldolase.

and his coworkers (MacGee and Doudoroff, '54). Compounds such as 2-keto-3-deoxygluconate 6-phosphate and 2-keto-3-deoxygalactonate 6-phosphate (Entner and Doudoroff, '52; De Ley and Doudoroff, '57) are cleaved to yield pyruvate and glyceraldehyde 3-phosphate (fig. 10). These reactions are essentially irreversible and each requires a different enzyme. Beyond this their mechanism is little understood. A new series of condensation reactions was disclosed when Srinivasan *et al.* (Srinivasan *et al.*, '55; Srinivasan and Sprinson, '59a, b) showed that phosphoenolpyruvate and erythrose 4-phosphate condense to form the 7-carbon precursor of shikimic acid (fig. 11). The dephosphorylated condensation product has been isolated and identified by Weissbach and Hurwitz ('59). Levin and Racker ('59) reported a similar condensation reaction with ribose 5-phosphate, which yields a 2-keto-3-deoxyoctonate 8-phosphate. The configuration of the C_8 sugar is not known.

Condensation of this type, involving phosphoenolpyruvate, may prove to be of great biological significance. Unlike the reaction with pyruvate as substrate, which appears to function biologically in the direction of cleavage, those in which phosphoenolpyruvate is the condensing agent have been demonstrated only in the direction of synthesis. Comb and Roseman ('58) reported the formation of *N*-

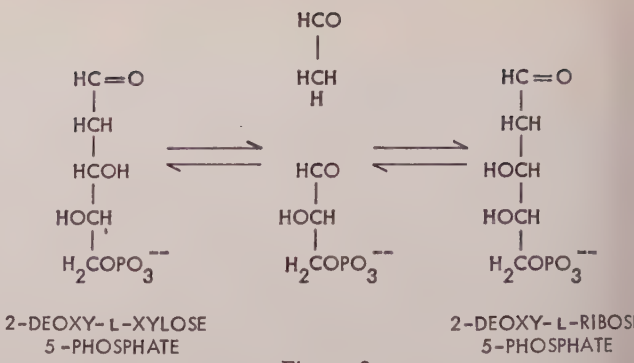

Figure 9

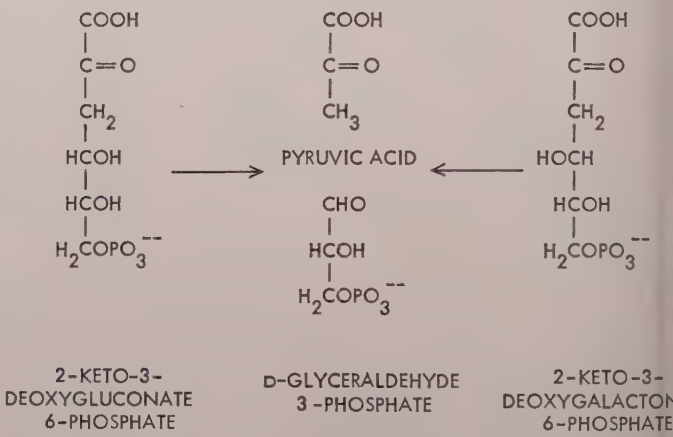

Figure 10

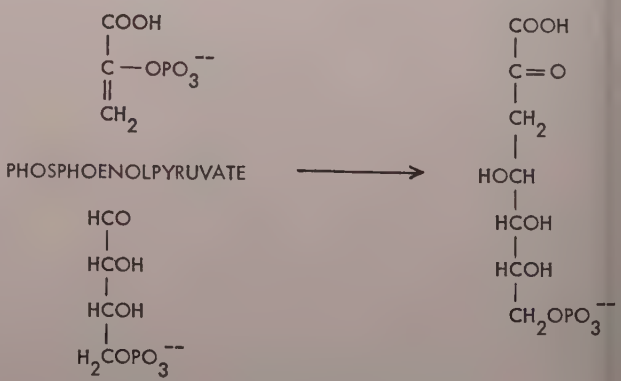

Figure 11

ylneuraminic acid (sialic acid) from
ctyl-D-mannosamine and pyruvic acid.
vever, the equilibrium favors the cleav-
reaction, and the biosynthetic pathway
y utilize phosphoenolpyruvic acid rather
n pyruvic acid.

ome light may be shed on the mech-
sm of condensation reactions involving
uvate by experiments now in progress
our laboratory. Pricer has found that
++ catalyzes a nonenzymic condensa-
involving aldehydes and the methyl
up of pyruvate, yielding compounds
t give the color test for 2-keto-3-deoxy-
onic acids. The nature and configura-
of the products are now under in-
tigation.

heptulose 7-phosphate. Transaldolase is highly specific for the substrates shown, although Racker and Schroeder ('57) have evidence for a reaction with ribose 5-phosphate to produce an octulose. Some reaction occurs with high concentrations (0.1 *M*) of free glyceraldehyde as acceptor.

This reaction differs from that catalyzed by fructose diphosphate aldolase mainly in that no dissociation of the dihydroxyacetone moiety occurs. Perhaps the carbanion-enol is in this case unable to react with proton from the aqueous phase. From Rose's exchange experiments with fructose diphosphate aldolase, it seems reasonable to conclude that the carbanion-enol reso-

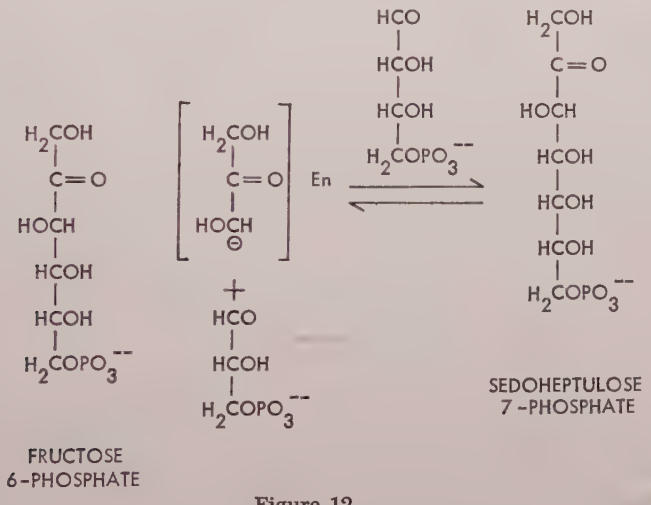

Figure 12

TRANSFER REACTIONS

Ve have considered simple condensa-
(or cleavage) reactions in which two
pounds combine to form one having a
ger carbon chain. In some cases, how-
, the cleavage product cannot be iso-
d as such; it forms a tight complex
n the enzyme, from which it is trans-
ed to another substrate.

ransaldolase. In the reaction catalyzed
transaldolase (Horecker and Smyrnio-
'55), an active dihydroxyacetone group
plit off, leaving free glyceraldehyde 3-
sphate (fig. 12). However, no reaction
be detected unless a suitable acceptor
resent, such as erythrose 4-phosphate,
which case the final product is sedo-

nance form dissociates less readily than the protonated molecule. Further experiments with stoichiometric quantities of purified transaldolase may provide a clue to the nature of the enzyme complex.

Transketolase. This enzyme catalyzes the transfer of C_2 units from ketoses such as D-xylulose 5-phosphate, D-fructose 6-phosphate, or sedoheptulose 7-phosphate (Horecker *et al.*, '53; Racker *et al.*, '54) (fig. 13). In this case cleavage occurs between the carbon atoms bearing the carbonyl group and the adjacent hydroxyl group and the enzyme is therefore a transketolase. The C_2 fragment, which corresponds to glycoladehyde, is not free but is bound to the enzyme and, for the reac-

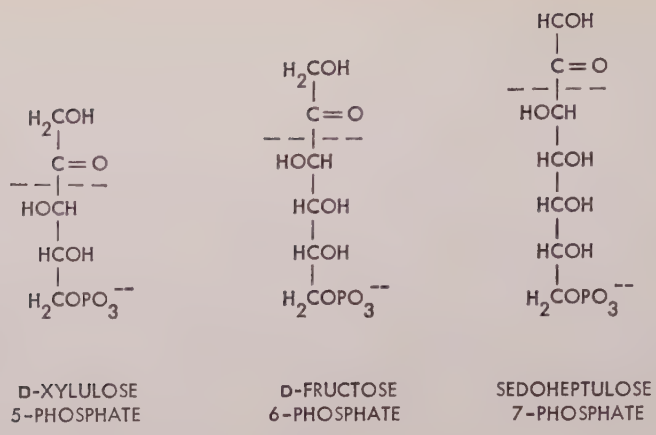

Figure 13

tion to proceed, a suitable acceptor must be present (fig. 14). The over-all reaction can therefore be represented as follows: with fructose 6-phosphate as substrate the products are tetrose phosphate and either sedoheptulose 7-phosphate or xylulose 5-phosphate, depending on whether ribose 5-phosphate or glyceraldehyde 3-phosphate is the acceptor. Any combination of substrate and acceptor can be used. A number of other aldehydes will serve as acceptors but have not been shown to be important biologically.

The coenzyme is thiamine pyrophosphate (Horecker and Smyrniotis, '53; Racker *et al.*, '53), and the available evidence suggests a glycolaldehyde-thiamine pyrophosphate complex is formed tightly bound to the enzyme since thiamine pyrophosphate itself does not serve as an acceptor.

Breslow has discussed his mechan (Breslow, '58) for the role of thiam pyrophosphate in this and other reactio

Phosphoketolase. The reaction cataly by phosphoketolase (Heath *et al.*, '58 superficially similar to that catalyzed transketolase, but there are a numbe important differences. This enzyme, wh has been detected in several pentose-

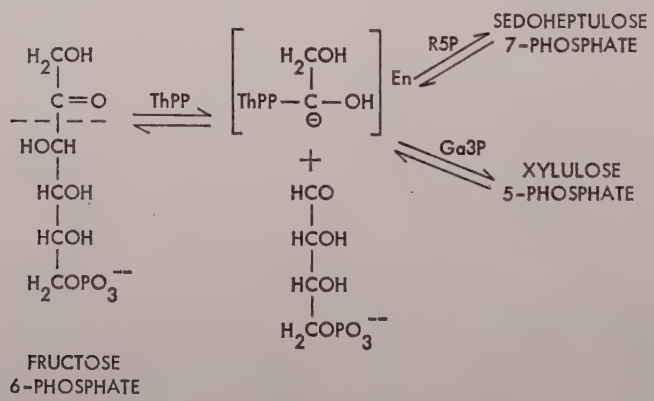

Figure 14

menting organisms (Heath *et al.*, Schramm and Racker, '57), converts x lose 5-phosphate to triose phosphate acetyl phosphate (fig. 15). A similar zyme that uses fructose 6-phosphate forms acetyl phosphate and tetrose p phate was found by Schramm and Rac ('57) in *Acetobacter xylinum*. The r tion requires inorganic phosphate and amine pyrophosphate, which is rea separated from the enzyme protein.

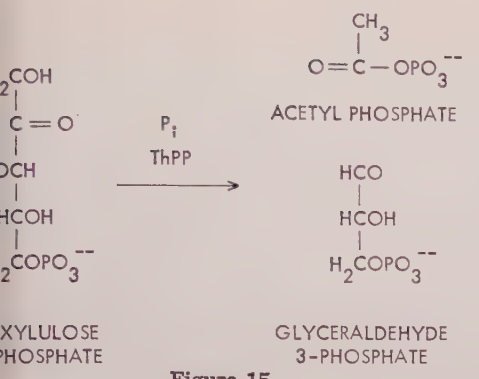

Figure 15

The facts impose several important re-
ctions on the reaction mechanism. In
first place, the reaction is not at all
ersible. No pentose phosphate is formed
m acetyl phosphate and glyceraldehyde
sphate nor can any exchange reactions
detected. Phosphate can be replaced
arsenate, in which case the product is
tate rather than acetyl phosphate, but
enzyme does not catalyze the arsenoly-
of acetyl phosphate or the exchange of
tyl phosphate with inorganic phosphate.
l more significant is the lack of ex-
nge with glyceraldehyde 3-phosphate,
n in the absence of inorganic phosphate.
s lack of exchange excludes triose phos-
te formation by a reverse ketol con-
sation such as may occur with trans-
olase and requires a different mech-
sm even for the first steps.

Lack of arsenolysis or exchange must
interpreted to mean that the last step,
sphorolysis of the acetyl group, is ir-
ersible and that the reaction involves a
e of intermediate that cannot be formed
m acetyl phosphate.

As in the aldolases the precise role of
enzyme in these reactions remains
known. The active forms of thiamine
ophosphate appear to remain attached
he enzyme, since no evidence has been
ained for thiamine pyrophosphate it-
as an acceptor of the C_2 groups, as
ght be expected if the complex dissoci-
d from the enzyme in the absence of
sphate. Phosphoketolase must some-
promote the removal of the negative
droxyl which was originally attached to
C-1 position.

The concerted action of transaldolase and transketolase on fructose 6-phosphate. From what we have seen of the requirements for transketolase and transaldolase neither enzyme should react to a measurable extent unless two substrates are added —one to act as donor and the other as acceptor for the enzyme-bound fragment. It was therefore difficult to understand an observation of Bonsignore and his coworkers ('57, '58), who found that the addition of fructose 6-phosphate alone to a thoroughly dialyzed liver extract gave rise to an immediate and rapid formation of sedoheptulose phosphate. This also has been reported by Dische ('58) with red cell preparations. This conversion is not caused by the oxidative steps, since 6-phosphogluconate is completely inactive. However, if the nonoxidative sequence catalyzed by transketolase and transaldolase is involved, then, in addition to fructose phosphate, an acceptor should be required to spark the reaction (Horecker and Mehler, '55). This process has now been studied by Pontremoli in our laboratory. To exclude the presence of possible acceptors formed from other components of the enzyme preparation, dialyzed liver extract was replaced by a mixture of spinach transketolase (Horecker *et al.*, '56) and yeast transaldolase (Horecker and Smyrniotis, '55), both highly purified (fig. 16). The rate of heptulose formation was exactly as rapid with equivalent quantities of the purified enzymes as with the crude liver

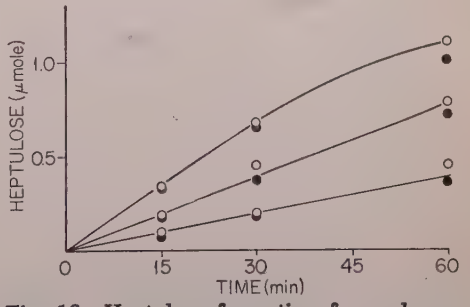

Fig. 16 Heptulose formation from glucose 6-phosphate with liver extract and purified enzymes. In the experiments with the dialyzed liver preparation the quantities used were 0.4, 0.2, and 0.1 ml, respectively. The purified enzymes were mixed to duplicate the activities found by analysis of the liver preparation. ○, With dialyzed liver supernate; ●, equivalent mixture: transketolase plus transaldolase.

extract. This result suggests that the reaction is indeed caused by a combined action of transketolase and transaldolase but fails to account for the fact that neither of these enzymes should have been able to initiate the reaction in the absence of a suitable acceptor. The reaction mixture in figure 16 included a muscle hexosephosphate isomerase preparation. In the absence of this enzyme (fig. 17), fructose

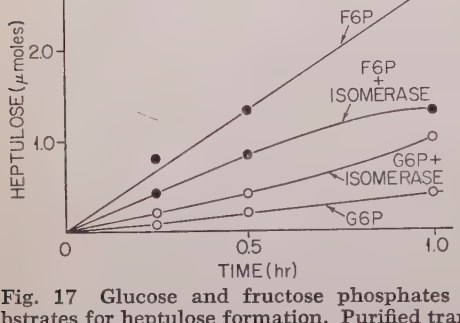

Fig. 17 Glucose and fructose phosphates as substrates for heptulose formation. Purified transketolase and transaldolase were used as in figure 16. A muscle hexosephosphate isomerase preparation was added as indicated.

6-phosphate is a far better substrate than glucose 6-phosphate, as is to be expected since the ketose ester and not the aldose ester is split by either transketolase or transaldolase.

As is seen in figures 16 and 17, the reaction begins at once on addition of fructose 6-phosphate or glucose 6-phosphate and no other substrate is needed. Stoichiometry is almost exactly that predicted from the transketolase-transaldolase sequence. If 1 mole of fructose phosphate is assumed to undergo a C_2–C_4 cleavage catalyzed by transketolase and a second mole of fructose phosphate to undergo a C_3–C_3 cleavage catalyzed by transaldolase, the first products would be heptulose phosphate and pentose phosphate (table 3). The lat would in turn be converted to one-h mole each of heptulose phosphate and ose phosphate. According to theory, each mole of fructose phosphate utili 0.75 mole of heptulose and 0.25 mole triose phosphate would be found. T amount of heptulose formed is in excell agreement with this prediction; the l yield of triose is accounted for by so residual pentose.

Two possible explanations must be c sidered to account for the reaction. On that the two enzymes interact in such way that each immediately provides acceptor needed by the other, at optim concentration. The second is that tra of an acceptor are present from the ginning and serve to prime the reacti Possible acceptors are glucose 6-phospha triose phosphate, or tetrose phosphate.

The second hypothesis will be cons ered first. Glucose 6-phosphate is rapi formed from fructose phosphate but, several grounds, it seems unlikely to act acceptor. In the first place, with fruct 6-phosphate as the substrate, a requi ment for glucose 6-phosphate in the ea part of the reaction cannot be dem strated; in fact, addition of isomer slows the reaction when fructose 6-ph phate is added (fig. 17). The over-all action proceeds as well with commerc fructose 6-phosphate, which contains nificant amounts of glucose 6-phospha as with a chromatographed sample wh is free of this ester. Under the conditi of these experiemnts, glucose 6-phosph is not an effective acceptor for either tra ketolase or transaldolase.

The presence of traces of tetrose ph phate or triose phosphate to initiate reaction can be excluded because (1) cose 6-phosphate, crystalline and free

TABLE 3

Stoichiometry in the conversion of hexose phosphate to heptulose phosphate

2 Fructose phosphate $\rightleftharpoons$ heptulose phosphate + pentose phosphate
Pentose phosphate $\rightleftharpoons$ 0.5 heptulose phosphate + 0.5 triose phosphate
Sum: 2 Fructose phosphate $\rightleftharpoons$ 1.5 heptulose phosphate + 0.5 triose phosphate

	Amount of heptulose or triose formed in indicated times per equivalent of fructose phosphate utilized					
	Theory	15 min	30 min	60 min	120 min	240 min
Heptulose phosphate	0.75	0.76	0.82	0.74	0.77	0.67
Triose phosphate	0.25	0.11	0.12	0.16	0.16	0.11

ıtaminants (Wood and Horecker, '53), ves as the sole substrate with no lag ase, and (2) the addition of triose or ose phosphate at zero time does not er the course of the reaction.

The alternative possibility is that each zyme splits one molecule of fructose 6- osphate, each thus producing the ac- otor for the other enzyme (fig. 18).

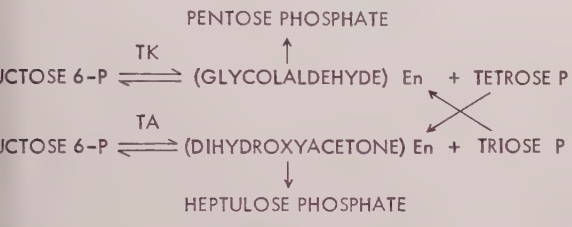

Figure 18

ı conventional kinetic grounds this is ghly unlikely, since initially neither tet- se phosphate nor triose phosphate would present in sufficient concentration. On e basis of reasonable assumptions re- rding molecular weight and purity, it n be calculated that the enzymes are esent in concentration not exceeding $^{-7}$ *M*. This sets an upper limit on the ıount of cleavage of fructose 6-phosphate at can occur in the absence of acceptor. ıus, in the cleavage of fructose 6-phos- ate by transketolase, the level of eryth- se 4-phosphate might reach 10^{-7} *M*. is is only one two-hundredth the K_m for

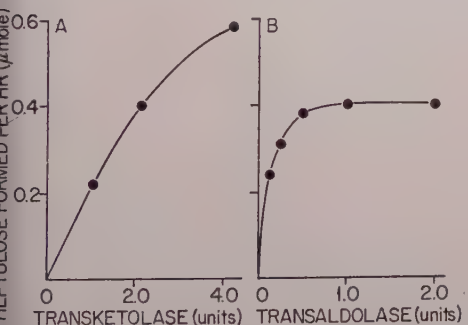

Fig. 19 Effect of levels of transketolase and nsaldolase on the rate of heptulose formation. e enzyme units are based in each case on ridine nucleotide oxidation or reduction and ı therefore be considered to be equivalent. ee Horecker *et al.*, '53; Horecker and Smyrnio- '55.) A: transaldolase, 1.0 unit. B: trans- olase, 2.1 units.

transaldolase, which is 2×10^{-5} *M*. Since an excess of triose phosphate is generated during the reaction, we would expect the reaction to show a definite lag phase, which would be overcome by triose or tetrose phosphate. This has not been observed.

On the other hand the reaction shows unusual aspects that do suggest an interaction of the two enzymes (fig. 19). Thus, in the presence of two units of transketolase, only a half unit of transaldolase is needed to saturate the over-all process. This is rather extraordinary. Usually, when the product formed by one enzyme is assayed with another, as when glucose 6-phosphate dehydrogenase is used to assay hexokinase, a large excess of the former enzyme is necessary. By contrast, even a fourfold excess of transketolase is insufficient to saturate transaldolase. This suggests that the reaction is somehow limited by transketolase and the limiting step is the removal of the bound C_2 unit.

From a teleological point of view, the remarkable efficiency of sedoheptulose formation from hexose monophosphate is not unexpected. This is the major biological mechanism for pentose synthesis (Horecker and Hiatt, '58) and as such should proceed smoothly and rapidly, without the necessity of accumulating large concentrations of the unstable compounds glyceraldehyde phosphate and tetrose phosphate. Whatever may be the interpretation of the observations, the fact remains that heptulose phosphate formation from hexose monophosphate will occur in the absence of detectable quantities of the triose and tetrose esters.

In summary, we have considered a few of the reactions involving C—C bond formation and cleavage by aldol and ketol condensations. The interesting acetoin condensations studied by Krampitz *et al.* ('58) have not been mentioned. Many other aldol condensations are worthy of note—several occur in the glyoxylate cycle of Kornberg and Krebs ('57), including the two reactions involving acetyl—CoA catalyzed by condensing enzyme (Stern *et al.*, '51) and malate synthetase (Wong and Ajl, '55; Kornberg and Krebs, '57). A more

typical aldol-type reaction in this series is the cleavage of isocitrate to succinate and glyoxylate. In the area of lipid synthesis, an interesting suggestion was made by Brady ('58), who proposed the condensation of a fatty acid aldehyde and malonyl —CoA. This brief summary still fails to exhaust the known biochemical reactions involving aldol and ketol condensations. Future developments in this area will be worth watching.

Finally, I would like to thank Mr. W. E. Pricer, Jr., and Drs. S. Pontremoli and A. Bonsignore for their valuable collaboration in the work reported.

OPEN DISCUSSION

BRESLOW[1]: The transketolase reaction requires a specific sort of catalyst. If the mechanism is written as a reverse aldol reaction, one of the moieties formed would be an anion with the negative charge sitting directly on a carbonyl rather than being next to it, and, therefore, this would not be expected to be a stable anion and the reaction would not go. Thiamine pyrophosphate stabilizes anions of this sort, and all the reactions in which it is involved can be written formally as involving intermediates of the type in which there is a minus charge on a carbonyl group. The function of thiamine pyrophosphate is to stabilize this species by forming a stable derivative.

I might just say in passing that this first anion (fig. 20) is related in a sense to cyanide ion. At any rate, this anion is readily available and can add to the c bonyl group to give the next intermedi If the reverse aldol-type reaction occur this point, the aldehyde group is lost we come to an anion that is no longer a carbonyl group but is an anion nex a thiazolium ring and is resonance sta ized. By reversing the original mechani and using a different aldehyde, we co have transferred the two-carbon piece fr one aldehyde to another. I think that t is the funtcion of thiamine pyrophosph in the transketolase reaction. Dr. Kra pitz has some evidence that these ded tions, which were made on studies model compounds, are probably cor enzymically.

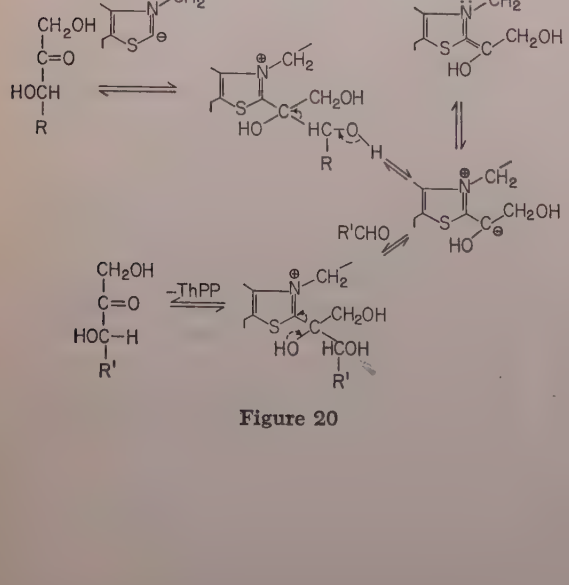

Figure 20

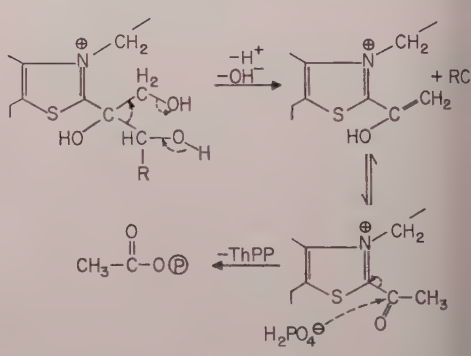

Figure 21

Figure 21 shows the sort of thing t has to be done in phosphoketolase. It tu out that the reaction is written simila to that for transketolase, but you reme ber the observation was made that ph phoketolase is not reversible with aldehyde portion. That is, if the ph phate is left out, this two-carbon pi cannot be transferred back onto aldehyde. So we must assume t either concertedly or perhaps i very rapid second step the anion t we used to have in the transketol reaction eliminates hydroxide i One difference is that this enzy must possess a group capable of vating the hydroxyl so that it is v rapidly eliminated. The two-car moiety is now an enol and it c of course, ketonize, and, if it d we get an acetyl group. This ac

[1] Ronald Breslow, Columbia Univers

ɔup should be "high energy" or very reac-
e, because we have here a carbonyl
ɔup sitting next to a C=N⊕. There is
y strong electrostatic repulsion of the di-
es, and this can probably be relieved
cleavage of this bond by attack of the
ɔsphate on the carbonyl. This would
enerate our thiamine anion.

KRAMPITZ[2]: As probably many of you
ɔw, S. Mizuhara and S. Mizuhara and
Handler showed that thiamine and py-
ate when adjusted to *p*H 8.5 formed
toin. This nonenzymic system resem-
s the acetoin-forming system obtained
m *Aerobacter aerogenes* in that α-aceto-
tic acid can be shown to be an inter-
diate in the formation of acetoin. For
ne time we have been interested in the
chanism of action of thiamine and have
that the nonenzymic system might help
elucidate the problem. Time does not
mit the description of experiments that
wed that neither the methylene group
ween the thiazole and pyrimidine rings
the sulfhydryl group of the open ring
ucture of the thiazole ring were involved
the mechanism of action. When we
ned of Dr. Breslow's suggestion and ex-
iments, which involved the active hy-
gen at position two of the thiazole ring
hiamine, we solicited the aid of Drs. J.
Sprague and C. S. Miller at Merck,
rp & Dohme to synthesize DL-3-[(2-
hyl-4-amino-5-pyrimidyl) methyl]-2-(1-
roxyethyl)-4-methyl-5-(2-hydroxyeth-
thiazolium chloride hydrochloride. We
e called the compound hydroxyethyl
mine, and it represents an α-hy-
xyethyl substitution at position two of
thiazole ring. The compound can be
ured as an addition of pyruvic acid to
tion two of the thiazole ring followed
decarboxylation. As already reported,
compound will replace thiamine in
itional experiments with *Lactobacillus*
enti. The compound with an ATP-
erating system was also active in re-
stituting a thiamine pyrophosphate-de-
nt yeast carboxylase preparation that
ained thiaminokinase. These results
be questioned on the basis that the
pound might be unstable under the
ting conditions, yielding free thiamine.
found, however, that the compound is
quite stable and remains intact under such
conditions.

Hydroxyethyl thiamine alone in the non-
enzymic system at *p*H 8.5 does not form
acetoin. When acetaldehyde is added to
serve as an acceptor, acetoin is formed,
and we have been able to demonstrate that
an acetoin complex with thiamine at posi-
tion two of the thiazole ring exists as an
intermediate.

Many years ago Bergel and Todd showed
that methyl thiamine, i.e., a methyl group
substituted at position two of the thiazole
ring, was nutritionally inactive. We also
synthesized this compound and showed
that it competitively inhibits the growth of
L. fermenti. Thiamine and hydroxyethyl
thiamine reversed the inhibiting action.

It is obvious that the pyrophosphate
derivative of hydroxyethyl thiamine is de-
sirable for further enzymic studies. We
have isolated this compound from incuba-
tion mixtures of acetaldehyde and thiam-
ine pyrophosphate at *p*H 8.5 and tested it
in the acetoin-forming system obtained
from *A. aerogenes*. As would be expected,
without an acceptor the compound will not
form acetoin. When pyruvate was added
to serve as an acceptor, acetoin was
formed. The concentration of pyruvate
used was such that only traces of acetoin
were formed from pyruvate alone.

The C_2 moiety of hydroxyethyl thiamine
pyrophosphate was transferred to pyru-
vate, in all probability forming acetolac-
tate. The latter was decarboxylated to
acetoin by the active acetolactic decarbox-
ylase in the enzyme preparation.

In preliminary attempts with yeast car-
boxylase, we have not been able to demon-
strate the formation of acetaldehyde from
hydroxyethyl thiamine pyrophosphate. We
are not able to account for this; however,
it may be that, in the absence of pyruvate,
the system is not furnished with the proper
acceptor for the formation of acetaldehyde.

BRESLOW: It actually works in the
acetoin system?

KRAMPITZ: Yes.

BROWN[3]: We have isolated this acetalde-
hyde-thiamine compound from *Escherichia
coli*, which contains surprisingly large

[2] L. O. Krampitz, Western Reserve University.
[3] G. M. Brown, Massachusetts Institute of Tech-
nology.

amounts of the phosphorylated form of the compound. One of the steps that led to the isolation of the compound included dephosphorylation by treatment with Takadiastase. The isolated compound was shown to be identical to the synthetic compound (which Dr. Sprague was kind enough to send us) by (1) its behavior on paper chromatograms in some seven different solvent systems, (2) its stability characteristics in acid and alkali, and (3) its growth-promoting properties for a variety of thiamine-requiring microorganisms.

GUNSALUS[4]: Not so much for the difficulties in the challenge as for their interest and importance, may I suggest a consideration of the other keto acid cleavages, particularly of pyruvate. Two types, referred to as clastic reactions, are known. In the final result, the electron pair shared by carbonyl and carboxyl appear not to be transferred with the carbonyl moiety. These acyl-generating cleavages occur predominantly in enteric bacteria (*E. coli*) and anaerobic bacteria (*Clostridia*) and differ with respect to the C_1 formed. The two types can be illustrated as in reactions (1) and (2) (fig. 22) in which R = OH,

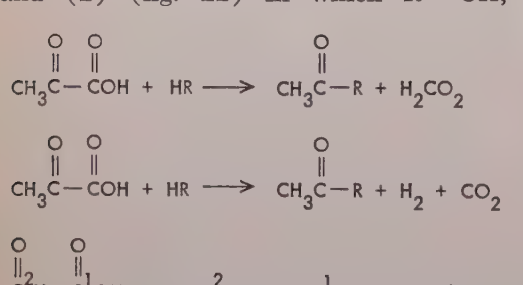

Figure 22

OPO_3H_2, or SCoA. These systems resemble yeast carboxylase and the pyruvate dehydrogenases of mammalian and microbial cells in the diphosphothiamine (DPT) requirements but are considered to differ from those in the generation of acyl without the intervention of an aldehyde-level oxidative product; i.e., the electron pair is considered to migrate with the carboxyl (C_1). A similar DPT-mediated cleavage of glyoxalate was observed in Barker's laboratory by Campbell ('54). This reaction (3) can be written as shown in figure 22.

In the presence of potassium hypoph
phite, glyoxalate yields carbon 2 as C
and carbon 1 as formate, whereas, in t
absence of hypophosphite, both carbo
from CO_2. Thus, from the products form
in the presence of hypophosphite, a s
cific inhibitor of formic dehydrogena
we may infer that carbon 2 does n
proceed via formate.

BRESLOW: I must admit that the clas
reactions represent a very serious proble
since as you point out, it is necessary
bring the electron pair along with t
carboxyl group to yield formic acid.
think it is probably fair to say that th
are only two serious possibilities. First
is possible that condensation of thiami
pyrophosphate occurs on the keto gro
of pyruvate in the usual way but that o
dation-reduction processes then occur
yield the clastic products. Second, it
possible that pyruvate is activated and th
condenses on its carboxyl end with
coenzyme. A simple extension of our ot
mechanisms would then lead to the clas
products. The product, a 2-formyl thia
lium salt, should be an "active" form
and could transfer its energy by w
known paths to activate pyruv
and complete the cycle.

I think this is really what
has to do. I must admit that I
not see anything that looks at
reasonable for these reactions t
does not involve either conden
tion on the carboxyl end or oxi
tion-reduction. I do not think
has to be modified very much
get CO_2 and hydrogen out. T
other system you mentioned has
be the same. Either there is n
mal condensation on the aldeh
group or the carboxyl is activated and c
denses itself. Since the reaction is a
oxidation, we must in either case inv
oxidation-reduction cofactors.

GUNSALUS: How would it strike you
go back in the other direction with
clastic pyruvate systems and assume
thiamine compound such as you sugges
for the pentulose phosphate cleavage
acetyl phosphate and triose phospha
Do you think such an intermediate i

[4] I. C. Gunsalus, University of Illinois.

ssibility? This would mean assuming
the clastic system that the carbonyl
oup attacks thiamine rather than car-
xyl as you have just suggested.

There are a number of experiments that
ight possibly furnish ground rules for
nsidering these mechanisms. Perhaps
e most pertinent are the data indicating
apid exchange of pyruvate carboxyl with
rmate in the *E. coli* system and with CO_2
the clostridial system. In the latter, the
esence of external electron acceptors of
gh potential, i.e., methylene blue or
racin, eliminates the CO_2 exchange.
qually important is the lack of evidence
r an exchange of the C_2, at least with any
preciable rate — and not for any lack
attempts to observe such.

The question here would perhaps be:
you visualize the formation of an acyl
iamine as possible; i.e., DPT serving as
carbonium-rather than carbanion-gener-
ing catalyst? Would a derivative similar
or identical with, the one indicated in
ure 21 in the generation of acetyl phos-
ate from ribulose be a suitable possibil-
?

Writing an oxidation-reduction reaction
the *E. coli* clastic system would obvi-
sly be difficult, for the clostridial CO_2H_2
stem possibly less so. I wonder if your
asoning should lead to further ques-
ning of your informants on the na-
e and firmness of the data ruling out
idation-reduction mechanisms. Among
demonstrated cofactors for the clostrid-
system, whose function is not all or
completely clarified, are ferrous iron,
unidentified $H_2 \leftrightarrow 2H$ cofactor, and evi-
nce that CoA may play a second role in
dition to its function as an acyl acceptor
d transfer agent.

BRESLOW: I am not sure I should specu-
e too much more here. The major prob-
n in making formic acid out of one of
se systems is how to get the hydrogen
o the carboxyl group. I mean, this is
thing that one expects thiamine to do.
en you see formic acid being formed,
n you feel that it is the carboxyl that
uld be attached to the thiazole ring.
The only other possibility that seems to
at all reasonable is some sort of reduc-
method to put hydrogen onto the car-
yl, and the possibility that there is any-
thing else involved here that will reduce carboxyls (some sort of oxidation-reduction confactor) is something that has to be kept in mind. I must admit that the type of thing you mention sounds very much as if the pyruvate is attached to the thiamine by the acetyl group, and, if this is so, I think there has to be some sort of oxidation-reduction process to get the hydrogen into the carboxyl.

Of course, there are often a fair number of chemically possible ways that we can write things about which there is not too much direct information. But, for instance, I am sure that everybody realizes that, if in some way the carboxyl group had been reduced to the equivalent of an aldehyde group, then cleavage in this kind of a system is also perfectly feasible. The thiazole ring is very useful in that it will stabilize two kinds of anions. It will stabilize anions on it and it will also stabilize anions next to it, and this is in fact the reason it is able to be a catalyst in this sort of cleavage.

TODD[5]: I wonder if I could ask for one piece of information about a matter that I was thinking about when Dr. Breslow was talking. Essentially, what he is saying is that 2-acetyl thiazolium, and presumably, by implication, 2-acetyl pyridinium, compounds are acetylating agents for ions. Has Dr. Breslow, in fact, any experimental evidence for this? It is not clear to me whether, in fact, he has done experiments with such acetyl compounds, or whether this is pure speculation at the moment.

BRESLOW: Well, I would say that we have not done anything with acetyl or pyrimidine compounds. In the thiazolium series, what I can say is that acetyl cyanide is an acetylating agent, and it seems that in the decomposition of the benzaldehyde derivatives of thiazolium salts one has evidence that the thiazolium ring is a pretty good leaving group. This sort of aldol reaction is not very much different from transferring an acetyl group, which is why I think these things will be acetylating agents. Presumably, the other problem then is whether it is really a very active, free acetylating agent. I must say that I

[5] Alexander Todd, University Chemical Laboratory, Cambridge, England.

do not have any direct evidence but I think it almost certainly is.

TODD: I think this is such an important matter that you ought to make an acetyl thiazolium compound to see whether it does, in fact, work as you suggest.

METZLER[6]: I want to comment on these reactions that give acetyl phosphate and formate. Isn't it quite reasonable to assume that condensation of the carboxyl group of pyruvate with a thiazolium Zwitter ion could occur if the carboxyl group were first converted to a thioester?

BRESLOW: Do you mean on the carboxyl?

METZLER: Yes, the thioester.

BRESLOW: Oh, yes, I think you can write that and you can regenerate the energies from the phosphorylysis of the formyl thiazolium derivative. It is just a little different from what one is used to having carboxyl do.

WOOD[7]: I think I would like to say something about transaldolase-exchange reactions in relation to isotope studies. In experiments on the C^{14} distributions in the galactose and glucose moieties of lactose, using glycerol 1,3–C^{14}, we have observed a very high activity in the bottom half of the galactose, i.e., in positions 4 and 6. We had anticipated that dihydroxyacetone phosphate would be the first product of the conversion of the glycerol and, if anything, the top half would be labeled the higher. Actually, the bottom half of the sugar was 10 times as hot as the upper half. We had never seen an isotope pattern like that in a hexose. The glucose moiety, on the contrary, was more like that expected. It was labeled somewhat higher in the upper half (C-1 and C-3) than in the lower half. This might be accounted for by a slow triose isomerase reaction.

To explain these results further I should give more information about the experiment. We injected the glycerol into the pudic artery of a cow (Wood *et al.*, '58). Thus the isotope went directly into the mammary gland and was converted into the milk product within the gland. In addition, of course, blood glucose was coming into the gland and was largely unlabeled. Without going into detail, we think that free glucose may be the precursor of the glucose moiety, and that the galactose moiety is formed from U galactose.

How can we account for the differe in isotope labeling that we observed? think that an exchange reaction may occurring in which transaldolase transf hot glyceraldehyde 3-phosphate aris from the glycerol directly into the bott half of the hexose phosphate. The bl glucose entering the gland has a low ac ity and is in part phosphorylated and c verted to fructose 6-phosphate. Then transaldolase exchanges the hot glyceral hyde 3-phosphate from the glycerol i the bottom half of the fructose 6-ph phate. The fructose 6-phosphate in t gives rise to UDP galactose while the g cose moiety comes from the free gluc which has not undergone the exchan The labeled blood glucose is proba formed in the liver and has a low activ a C^{14} pattern like the liver glycogen.

Recently, with Racker and Cou (Wood *et al.*, '59), we tested the transal lase exchange directly. Racker had so hot carboxyl-group-labeled phosphogly ate from his photosynthetic studies. T used phosphoglycerate 1-C^{14}, ATP, ph phoglycerate kinase, triosephosphate de drogenase, DPNH, Mg^{++}, fructose 6-ph phate, and transaldolase. There was change in the amount of fructose 6-ph phate, and by using a large amount fructose 6-phosphate with a small amo of phosphoglycerate, about 80% of the was transferred into the fructose 6-ph phate. We degraded the fructose 6-ph phate and all the isotope was in the position. We are not certain that transaldolase exchange is the explana of our results with the cow but it certa fits in with our observations.

I may say that this is really an extren nice way to make 4-labeled hexose. Labeled glucose has been available, once in a while it would be nice to ha 4-labeled sugar. In addition, I see reason why 5-labeled sugars could no made, starting with glycerol 2-C^{14}.

HORECKER: I agree that the transa lase exchange is probably one explana for the asymmetric labeling, but I thinl

[6] D. E. Metzler, Iowa State College.
[7] H. G. Wood, Western Reserve Universit

ould not exclude that suggested by rkes and tested by Rose ('58b), namely, at there is a rapid equilibration of these o trioses but, in the presence of a large nount of fructose diphosphate formed m unlabeled glucose, there can be a pid exchange of glyceraldehyde phos- ate with the bottom half of the fructose osphate molecule — five times as rapid the exchange of dihydroxyacetone phos- ate. This is simply the exchange cata- ed by adolase. Both types of exchange ld the same result.

WOOD: I think that is true, but I prefer nsaldolase exchange because we found ch very large differences.

HORECKER: The other would give you % at the maximum.

WOOD: We found more than that. Both ay be working; I see no reason why they ould not. I think it is obvious that these change reactions may throw complica- ns into the interpretation of isotope udies and, of course if both transketolase d transaldolase shuffle the C^{14} around, e may have a very complicated picture.

MCRORIE[8]: I should like to mention efly some of our results on the substrate ecificity of aldolase that we have ob- rved in our studies on the utilization of onic acids by microorganisms, where eavage of 1-phosphoketuronic acid occurs th fructuronic phosphate and tagaturo- e phosphate. Using muscle aldolase, we und that, of course, fructose diphos- ate is well cleaved and that fructuronic id 1-phosphate is cleaved slowly by mus- aldolase. This enzyme(s) is also con- tutive in microorganisms. If, however, e organisms are cultured on galacturonic d, the aldolase activity toward the 1- osphoketuronic acids is very much gher than in the case of the constitutive zymes.

This could arise, of course, from two ssibilities, the adaptation of a new aldo- e or the modification of the existing nstitutive aldolase. Since we have been mpered in our isolation and assay, in e study of reverse reaction, by the un- ailability of the phosphorylated keturo- acids and of the tartronic semialde- de, we have purified these enzymes ing fructose diphosphate as a substrate. r results indicate that there is a possible modification of the existing constitutive aldolase rather than the adaptation of a new aldolase in the presence of the phosphoketuronic acids.

[8] R. A. McRorie, University of Georgia.

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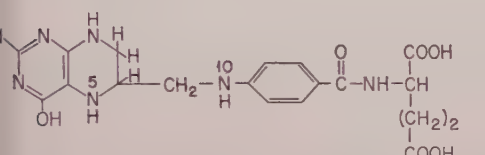
echanisms of Formylation and Hydroxymethylation eactions[1]

F. M. HUENNEKENS, H. R. WHITELEY, AND M. J. OSBORN[2]
Departments of Biochemistry and Microbiology, University of Washington, Seattle

The metabolism of one-carbon (C_1) ts mediated by folic acid coenzymes become better understood at the molec- r level owing to the ever-increasing essibility of the pertinent enzymes and nzymes. Five different C_1 structures at oxidation levels of formaldehyde and mate bound to tetrahydrofolic acid (fig. have been encountered in living sys- ns.[3] Formyl, formimino, and hydroxy- thyl groups form single covalent link- s with either the 5 or 10 positions of



TETRAHYDROFOLIC ACID

C=O	HC=NH	CH	DERIVATIVES OF HCOOH
ORMYL	FORMIMINO	METHENYL	
$_2$COH		CH_2	DERIVATIVES OF HCHO
ROXYMETHYL		METHYLENE	

ig. 1 Structure of tetrahydrofolic acid and groups.

rahydrofolic acid; the methenyl and thylene groups are bridged to both itions. The generation, transformation, l utilization of these "active" C_1 units by ious enzyme systems are summarized figure 2. The occurrence of such an egrated metabolic network for C_1 metab- m was suggested earlier by tracer ex- iments, which disclosed a facile trans- of C^{14} label between certain carbon ms, notably the β-carbon of serine, the and C-8 positions of purines, the C-2 ition of histidine, the methyl groups of thionine and thymine, and free HCOOH l HCHO.

The role of folic acid in these reactions has been reviewed elsewhere (Welch and Nichol, '52; Sakami, '55; Greenberg and Jaenicke, '57; Huennekens *et al.*, '58; Huennekens and Osborn, '59; Buchanan and Standish, '59). In this paper we will consider mechanisms for the various formylation and hydroxymethylation reactions. These mechanisms have been deduced from existing descriptive data, such as kinetic and thermodynamic constants, cofactor requirements, and detection of intermediates; they also draw upon known mechanisms for analogous enzymic and chemical reactions. It must be pointed out, however, that not all reactions in figure 2 are at present equally suitable for this approach, and few of these reactions have been studied with O^{18}-, H^2-, or P^{32}-labeled substrates. Consequently, decisive information about the sites of bond cleavage and formation is still lacking.

REACTION MECHANISMS INVOLVING "ACTIVE FORMATE"

De novo synthesis of "active formate"

Formate-activating enzyme. The *de novo* synthesis of "active formate" from its components, formate and tetrahydro-

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[2] Present address: Department of Microbiology, New York University.

[3] This review will be limited to C_1 reactions that are mediated by tetrahydrofolic acid. Thus reactions involving HCHO and HCOOH in the free state (e.g., the DPN-linked oxidation of HCHO to HCOOH, or HCOOH to CO_2) or reactions requiring C_1 compounds "activated" by interaction with coenzymes other than tetrahydrofolic acid (e.g., carbonyl biotin, *S*-adenosyl methionine, and the CoA-dependent activation of formate) will be omitted.

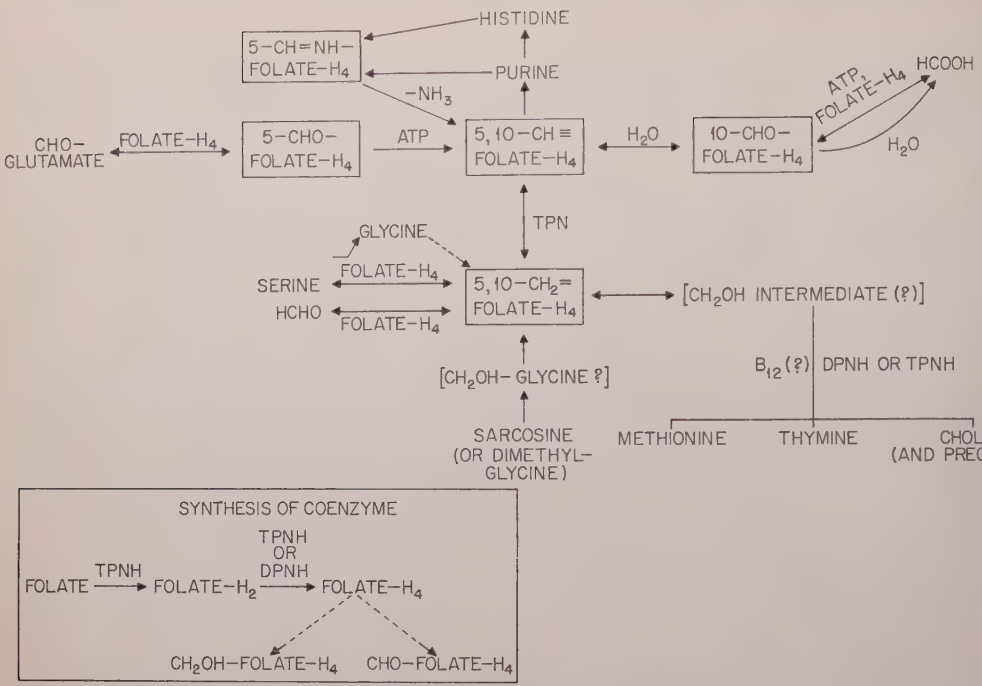


Fig. 2 Metabolic interrelations. Folate-H_4, 5,6,7,8-tetrahydrofolic acid. The prefixes (with their proper bonds) are as follows: **CHO**, formyl; **CH = NH**, formimino; **CH ≡**, methenyl; **CH_2=**, methylene; **CH_2OH**, hydroxymethyl.

$$\text{HCOOH + ATP + TETRAHYDROFOLATE} \xrightleftharpoons{Mg^{++}} \text{N}^{10}\text{-FORMYL TETRAHYDROFOLATE + ADP + P}_i \quad (1)$$

$$\text{ATP + TETRAHYDROFOLATE} \rightleftharpoons \text{PHOSPHORYL TETRAHYDROFOLATE + ADP} \quad (2)$$

$$\text{HCOOH + PHOSPHORYL TETRAHYDROFOLATE} \rightleftharpoons \text{N}^{10}\text{-FORMYL TETRAHYDROFOLATE + P}_i \quad (3)$$

Figure 3

folic acid, is a typical example of amide synthesis except that the acid is somewhat stronger ($pK_a = 3.7$) than other aliphatic carboxylic acids ($pK_a \cong 4.7$) and the base is a sterically hindered secondary amine. Under chemical catalysis, an acidic medium and a dehydrating agent are required to form the desired C—N bond. The formate-activating enzyme (also called "tetrahydrofolate formylase") catalyzes this reaction under more gentle conditions and ATP supplies the driving force reaction (1), fig. 3; P_i = inorganic or phosphate]. This enzyme has been pur from pigeon liver (Greenberg and nicke, '57; Jaenicke, '58) and from *M coccus aerogenes* (Whiteley, Osborn, Huennekens, '59), and crystallized f *Clostridium cylindrosporum* (Rabino and Pricer, '58; Rabinowitz, '58).

Studies with the *M. aerogenes* (W ley, Osborn, and Huennekens, '58; W

et al., '58, '59) and avian liver (Jaeke, '58) enzymes have suggested that three-component reaction (1) proceeds separate steps [reactions (2) and (3), 3]. This formulation rests upon the lowing experimental observations:

(a) Formate-activating enzymes have en partially purified from a wide variety living systems, e.g., microorganisms, tebrate and invertebrate animals, and nts (H. R. Whiteley, unpublished data), l in all cases, the enzyme catalyzes not y the over-all reaction (1), as measured ner by N^{10}-formyl tetrahydrofolate or P formation,[4] but also the initial reac- n (2), as measured by ADP formation m ATP and tetrahydrofolate (Whiteley, born, and Huennekens, '58; Jaenicke,). In the latter instance N^{10}-formyl rahydrofolate is not found, thus proving t none of the reactants are contamted with HCOOH. It should be noted t the amount of enzyme required to monstrate reaction (2) is much larger n that required for reaction (1), indiing perhaps that phosphoryl tetrahydroate is enzyme bound.

(b) During the enzyme-catalyzed interion of ATP and tetrahydrofolate, the ctrum of the latter (λ_{max} at 298 mμ) is laced by that of a new compound Whiteley, Osborn, and Huennekens, '58; nicke, '58) having an absorption maxim at the same wave length but with ower extinction coefficient. The extent this spectral change depends on the ount of enzyme, again suggesting that intermediate (phosphoryl tetrahydroate) is enzyme bound. Addition of mate results in the spectrum of the ermediate being replaced by that of -formyl tetrahydrofolate.

(c) Exchange of P^{32} into ATP occurs y in the presence of the complete sys-, i.e., enzyme, formate, ATP, and tetrarofolate (Greenberg and Jaenicke, '57; nicke, '58; Whiteley *et al.*, unpublished a).

(d) A compound having the properties formyl phosphate (Greenberg and nicke, '57) does not substitute for ATP l formate in reaction (1), nor is the r-all reaction inhibited by hydroxyline.

(e) Incubation of ATP^{32} with tetrahydrofolate in the presence of enzyme yields a fluorescent, P^{32}-labeled substance (Greenberg and Jaenicke, '57; Jaenicke, '58; Whiteley, Osborn, and Huennekens, '58; Whiteley *et al.*, '58) that can be isolated from the reaction mixture by chromatographic methods. This material, presumably phosphoryl tetrahydrofolate, when added to ADP in the presence of enzyme, yields small, but detectable, amounts of ATP and tetrahydrofolate, whereas in the presence of formate and the enzyme, N^{10}-formyl tetrahydrofolate and P_i are produced.

(f) Treatment of tetrahydrofolate with P_2O_5 and $H_3P^{32}O_4$ yields, in addition to degradation products, a small amount of a fluorescent, P^{32}-labeled material (Whiteley *et al.*, '58) that can be separated from the mixture by paper chromatography and is reactive in reactions (2) and (3). Chemical synthesis of a phosphorylated derivative of tetrahydrofolate has also been reported by Jaenicke ('58). Isolation of chemically or enzymically synthesized phosphoryl tetrahydrofolate is difficult because the N—P linkage is unstable in both acid and base and the compound is highly susceptible to air oxidation owing to the unprotected tetrahydropyrazine ring. Studies on the chemical synthesis and properties of model compounds related to phosphoryl tetrahydrofolate are in progress (J. G. Ozols and P. T. Talbert, unpublished data).

(g) Differential inhibition of the two steps has been achieved by treating the *M. aerogenes* and avian liver enzymes with *p*-chloromercuribenzoate (Jaenicke, '58; Whiteley *et al.*, unpublished data). It has not been possible to separate by physical methods the *M. aerogenes* enzyme into fractions responsible for each step.

If reaction (1) is carried out as outlined in reactions (2) and (3), a reasonable mechanism would be that shown in figure 4. In step I, the nucleophilic attack by the unshared electrons of the N^{10} atom on the

[4] N^{10}-formyl tetrahydrofolate is determined by conversion to N^5,N^{10}-methenyl tetrahydrofolate (λ_{max} at 355 mμ) after acid deproteinization, and ADP is estimated by DPNH disappearance in the combined pyruvate kinase-lactic dehydrogenase assay.

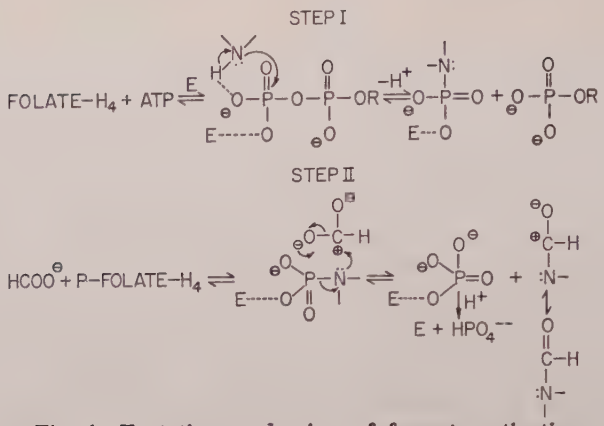


Fig. 4 Tentative mechanism of formate activation.

positive center of the polarized, terminal P—O bond of ATP is consistent with mechanisms postulated for many other ATP-dependent reactions (Kornberg, '57). The mechanism of the second step might be written in several different ways. For example, the phosphate group could be released as formyl phosphate, which, in turn, could react with the free N^{10} atom to form N^{10}-formyl tetrahydrofolate. However, such a mechanism involving formyl phosphate as an intermediate (unless it were enzyme bound and shielded) would appear to be unlikely inasmuch as the over-all reaction is not inhibited by high concentrations of hydroxylamine. Alternatively, we may propose the formulation given in step II of figure 4, where the enzyme is visualized as promoting the otherwise difficult shift of electrons leading to the expulsion of the phosphoryl group by the incoming polarized formyl group. This mechanism, moreover, would predict the migration of O^{18} from the carboxyl group to the phosphate group during the over-all reaction; such an effect has been observed in the analogous reaction for succinate activat (Cohn, '51; Hager, '57).

The enzymic activation of most carb ylic acids proceeds via an acyl adenyl intermediate and results in the format of AMP and PP as end products (Be '55; Ingraham and Green, '58). A sec type of activation yields ADP and P_i, the nature of an intermediate, if any, not been elucidated in all instances. formate- and succinate-activating enzy and glutamine synthetase apparently within the second category. The seque in reactions (2) and (3) is analogous that postulated for the succinate-activat enzyme from *Escherichia coli* (Smith *et* '57; Gunsalus and Smith, '58) [reacti (4) and (5), fig. 5]. The reactions catalyzed by separate enzyme fracti On the other hand, glutamine synthet which carries out reaction (6) (fig. 5) atypical in that a phosphorylated in mediate does not appear to be invol (Meister, '57).

Isomerization of folinic acid. A sec pathway leading to the formation of

$$\text{ATP} + \text{CoA} \rightleftharpoons \text{S-PHOSPHORYL CoA} + \text{ADP} \qquad (4)$$

$$\text{S-PHOSPHORYL CoA} + \text{SUCCINATE} \rightleftharpoons \text{SUCCINYL CoA} + P_i \qquad (5)$$

$$\text{GLUTAMATE} + \text{ATP} + NH_3 \rightleftharpoons \text{GLUTAMINE} + \text{ADP} + P_i \qquad (6)$$

Figure 5

$$N^5\text{-FORMYL TETRAHYDROFOLATE} + ATP \longrightarrow N^{10}\text{-FORMYL TETRAHYDROFOLATE} + ADP + P_i \quad (7)$$

$$N^5\text{-FORMYL TETRAHYDROFOLATE} + ATP + H^+ \longrightarrow (N^5,N^{10}\text{-METHENYL TETRAHYDROFOLATE})^+ + ADP + P_i \quad (8)$$

$$(N^5,N^{10}\text{-METHENYL TETRAHYDROFOLATE})^+ + H_2O \rightleftharpoons N^{10}\text{-FORMYL TETRAHYDROFOLATE} + H^+ \quad (9)$$

Figure 6

myl tetrahydrofolate involves the ATP-pendent, unidirectional isomerization reenberg, '54) of folinic acid (N^5-formyl rahydrofolate) [reaction (7), fig. 6]. is reaction, catalyzed by folinic isomer-, yields the same end products as reac-n (1) but differs in that the formyl group already attached to the N-5 position of rahydrofolate.

There is considerable doubt about the tus of the intermediate, or interme-tes, in reaction (7). In one system, a tially purified fraction from *M. aero-es* (Kay *et al.*, '59), the transient ap-rance of N^5,N^{10}-methenyl tetrahydro-ate suggests the sequence in reactions and (9) (fig. 6). Reaction (9) is own to be catalyzed by the enzyme cyclo-drolase (Rabinowitz and Pricer, '56); , in fact, this activity could be demon-ted in the bacterial folinic isomerase. second system, found in sheep liver parations (Peters and Greenberg, '57,), carries out the isomerization presum-by reactions analogous to (8) and , but the intermediate formed is similar ut not identical with, the known forms N^5,N^{10}-methenyl tetrahydrofolate (Cosu- *et al.*, '51, '52). In a third system, purified from chicken liver (Kay *et al.*, '59), neither cyclohydrolase activity nor N^5,N^{10}-methenyl tetrahydrofolate formation can be demonstrated during the over-all conversion of N^5- to N^{10}-formyl tetrahydrofolate.

Reactions (8) and (9) adequately describe the bacterial system (and may be applicable as well to the other systems), and the occurrence of a bridge compound in the sequence is analogous to the formation of the cyclic intermediates in the migration of phosphoryl and acyl groups. Reaction (8) is probably more complex than indicated and may involve a phosphorylated intermediate. If the mechanism proposed for formate activation is extended to the present reaction, the phosphate group would be located transiently on the N-10 position and subsequently displaced by the N^5-formyl group. A second mechanism for the isomerization could also be suggested. It is known that N^5-formimino tetrahydrofolate is deaminated to N^5,N^{10}-methenyl tetrahydrofolate in the absence of ATP by the enzyme cyclodeaminase (Rabinowitz and Pricer, '56) [reaction (10), fig. 7]. Similarly, in reactions (8) and (9), ATP might interact

$$N^5\text{-FORMIMINO TETRAHYDROFOLATE} + 2H^+ \longrightarrow (N^5,N^{10}\text{-METHENYL TETRAHYDROFOLATE})^+ + NH_4^+ \quad (10)$$

$$\text{FORMYL TETRAHYDROFOLATE} \longrightarrow HCOOH + \text{TETRAHYDROFOLATE} \quad (11)$$

Figure 7

with the N^5-formyl group to produce an intermediate of the type shown in figure 8, followed by expulsion of the phosphate group by the N^{10} atom.

Figure 8

Hydrolysis of "active formate"

Enzymic deacylation of N^{10}-formyl tetrahydrofolate. Although both the N^5- and N^{10}-formyl derivatives of tetrahydrofolate are susceptible to chemical hydrolysis, the only known enzyme-catalyzed reaction of this type [reaction (11), fig. 7] is specific for N^{10}-formyl tetrahydrofolate. The N^{10}-formyl tetrahydrofolate deacylase from beef liver (Osborn, Hatefi, *et al.*, '57) requires the presence of catalytic amounts of TPN or TPNH; DPN or DPNH are ineffective. The pyridine nucleotide may be required only to maintain an enzyme-bound sulfhydryl group in the reduced state, and in this event, the mechanism deacylation may resemble ordinary aci base catalysis of amides (see fig. 9). symmetrical intermediate, shown in br ets, has been invoked to explain the servation that O^{18} is exchanged from H into unreacted benzamide during its drolysis in base; a similar exchange also been noted in the base-catalyzed drolysis of esters. During the acid hyd ysis of benzamide, no O^{18} exchange is served (Bender and Ginger, '55), whe exchange still occurs in acid hydrolysi esters. An analogous mechanism for enzymic hydrolysis for N^{10}-formyl te hydrofolate is shown in figure 9B.

Thermodynamic and kinetic data for hydrolysis of formyl derivatives of te hydrofolate. The formyl derivatives tetrahydrofolate are chemically inter vertible [reaction (12), fig. 10], and ti conversions provide useful thermodyna and kinetic information for interpre the related enzyme-catalyzed transfor tions shown in figure 2. The three ami

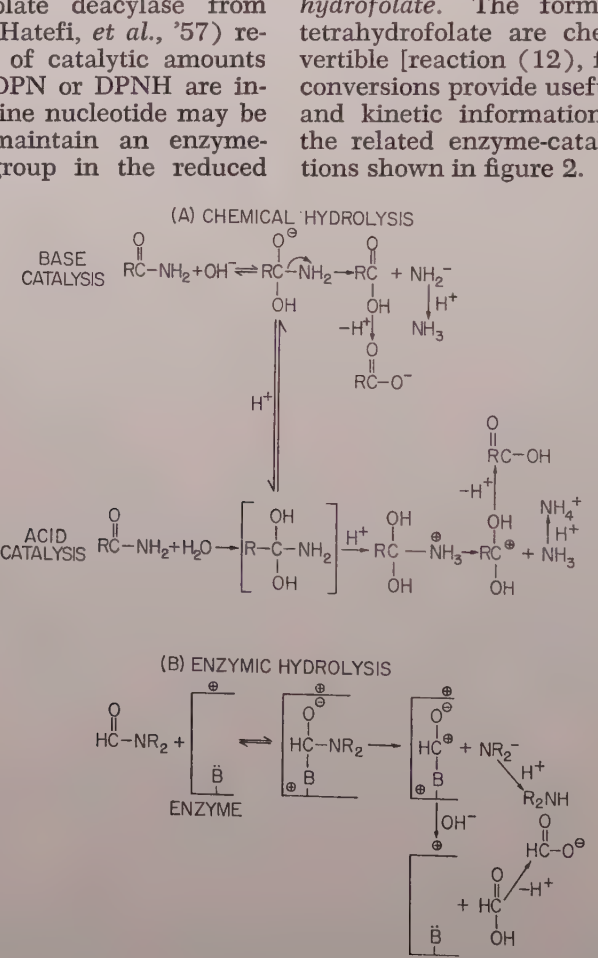


Fig. 9 Mechanisms for chemical and enzymic hydrolysis of amides.

- and N^{10}-formyl tetrahydrofolate and ,N^{10}-methenyl tetrahydrofolate, have dif- rent free energies for hydrolysis into rahydrofolate and formate. Of the var- us forms of "active formate," N^5- and ^{0}-formyl tetrahydrofolate are simple nides, whereas N^5,N^{10}-methenyl tetra- drofolate may be considered as an ortho- nide from which water has been removed g. 11). Kay *et al.* ('59) calculated that e $-\Delta F°$ values are in the relative order: ,N^{10}-methenyl tetrahydrofolate > N^{10}- rmyl tetrahydrofolate >> N^5-formyl trahydrofolate. It is significant that the lue for N^{10}-formyl tetrahydrofolate is 4 al/mole larger than that of N^5-formyl trahydrofolate. Using the data obtained r the synthesis of *N*-formyl glutamate eaction (14), fig. 12; Silverman *et al.*, '57], we may estimate that the $\Delta F°$ value for N^5-formyl tetrahydrofolate is somewhat lower (perhaps $\Delta F° = -2$ kcal/mole) than that of simple peptides for which values of 3–4 kcal/mole have been assigned (Borsook, '54). On this basis, the $\Delta F°$ value for the hydrolysis of N^{10}-formyl tetrahydrofolate would be ~ -6 kcal/mole, or ~ 1–2 kcal/mole less than ATP. This is consistent with the observation that reaction (1) (see fig. 3) proceeds readily in the forward direction.

As outlined in the tentative mechanism in figure 13A, the chemical conversion of N^5- or N^{10}-formyl tetrahydrofolate to N^5, N^{10}-methenyl tetrahydrofolate [see also reaction (12), fig. 10] requires acid; these processes are reversed in base. At neutral *p*H, phosphate ion accelerates the inter-

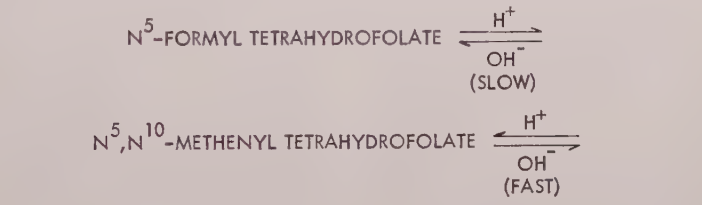


Figure 10

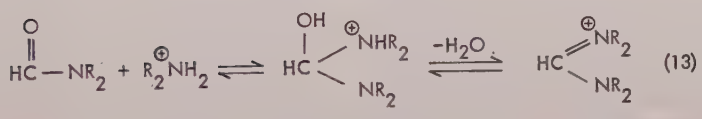
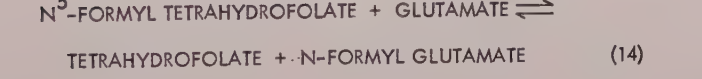


Figure 11



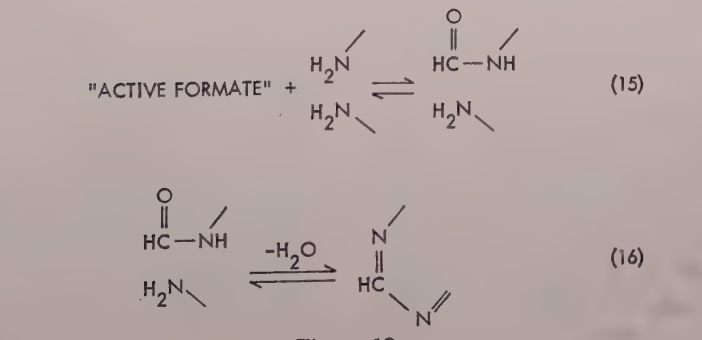


Figure 12

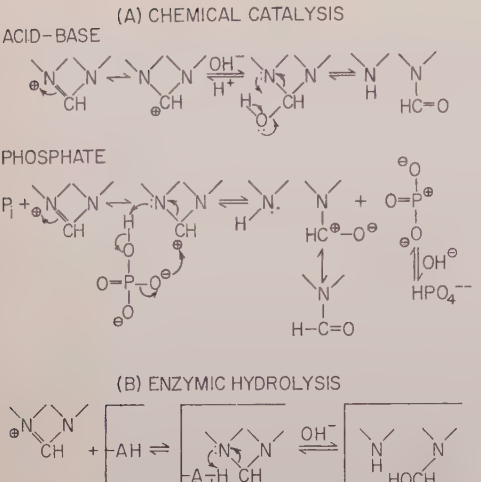


Fig. 13 Tentative mechanisms for the N^5,N^{10}-methenyl tetrahydrofolate $\rightleftharpoons$ N^{10}-formyl tetrahydrofolate conversion.

conversion rate of N^{10}-formyl tetrahydrofolate and N^5,N^{10}-methenyl tetrahydrofolate (Rabinowitz and Pricer, '56); an explanation for this effect is provided also in figure 13A. The enzyme cyclohydrolase may also utilize the same device, i.e., adjacent basic and acidic groups, to catalyze this reaction (fig. 13B). No enzyme for the analogous interconversion of N^5-formyl tetrahydrofolate and N^5,N^{10}-methenyl tetrahydrofolate is known, although, as discussed earlier, the ATP-dependent folinic isomerase may form N^5,N^{10}-methenyl tetrahydrofolate as an intermediate in reaction (7) (see fig. 6).

Amide exchange involving "active formate"

The reversible formylation of glutamic acid by N^5-formyl tetrahydrofolate (Silverman *et al.*, '57), according to reaction (14), is an example of amide exchange where one base is replaced by another. Counterparts to this reaction are found in transpeptidations (Borsook, '54) and in the glutamine synthetase-catalyzed change of hydroxylamine or hydrazine the amide group of glutamine (Meist '57).

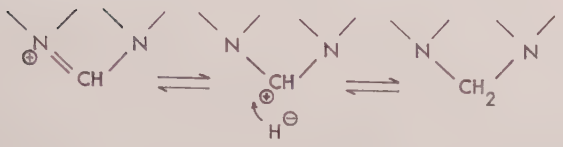
Other examples of amide exchange those involving the formylation of: (aminoimidazolecarboxamide ribonucl tide to yield inosinic acid or (b) glyc amide ribonucleotide to yield aminoimi zole ribonucleotide (Buchanan, '5 Either N^{10}-formyl tetrahydrofolate or N^{10}-methenyl tetrahydrofolate (but not formyl tetrahydrofolate) can serve as formyl donor for these reactions; cyc hydrolase in the preparations may accou for the equivalence of the two forms "active formate." In both reactions ri closure probably occurs in two steps actions (15) and (16), fig. 12]. For aminoimidazolecarboxamide ribonucl tide → inosinic acid conversion, ami imidazolecarboxamide ribonucleotide tra formylase and inosinicase catalyze re tions comparable to (15) and (16). more-complex sequence is apparently volved in glycinamide ribonucleotide aminoimidazole ribonucleotide conversi where a reaction similar to (15), leading formyl glycinamide ribonucleotide, is s sequently followed by an ATP-depende ring closure.

The amide exchange reactions (14) a (15) probably involve a mechanism si ilar to that presented in figure 9, exc that the base, H_2O, is replaced by an ami group. Reaction (16) is another exam of orthoamide formation.

INTERCONVERSION OF "ACTIVE FORMAT AND "ACTIVE FORMALDEHYDE"

Hydroxymethyltetrahydrofolic dehyd genase (Osborn and Huennekens, '5 catalyzes the interconversion of "act formate" and "active formaldehyde" cording to reaction (17) (fig. 14). The hydrogenase reaction is interesting in much as the "onium" structure of N^5,N methenyl tetrahydrofolate is conserv through the creation of a similar structu in TPN. Most reactions involving pyridi nucleotides and their substrates proceed way of a hydride ion mechanism (Mah and Douglas, '57; Westheimer, '59). hydride ion mechanism may also be w ten for the oxidoreduction of the bound

$(N^5,N^{10}\text{-METHENYL TETRAHYDROFOLATE})^+ + TPNH \rightleftharpoons$
$N^5,N^{10}\text{-METHYLENE TETRAHYDROFOLATE} + TPN^+$ (17)



(18)

HCHO + TETRAHYDROFOLATE $\rightleftharpoons$ "ACTIVE FORMALDEHYDE" (19)

Figure 14

gment in reaction (18) (see fig. 14). s, therefore, not surprising to find that ohydride is the most effective agent for chemical reduction of N^5,N^{10}-meth-l to N^5,N^{10}-methylene tetrahydrofolate ennekens and Osborn, '58).

REACTION MECHANISMS INVOLVING "ACTIVE FORMALDEHYDE"

Synthesis and degradation of "active formaldehyde"

The reversible synthesis of "active form-ehyde" from tetrahydrofolate and HO, as shown in reaction (19) (fig.), can be carried out chemically (Green-g and Jaenicke, '57; Kisliuk, '57; Blak-'57; Osborn *et al.*, unpublished data) enzymically (Osborn, Vercamer, *et al.*,). The structure of "active formalde-e" (Huennekens *et al.*, '58) is believed be N^5,N^{10}-methylene tetrahydrofolate reenberg and Jaenicke, '57; Kisliuk, '57; kley, '58; Huennekens and Osborn, '58) the basis of the following lines of dence.

a) When the hydroxymethyl tetrahy-folic dehydrogenase [reaction (17), fig. is freed from cyclohydrolase, N^5,N^{10}-thenyl tetrahydrofolate (but not N^5- or formyl tetrahydrofolate) can be re-ed to "active formaldehyde" and, con-sely, only N^5,N^{10}-methenyl tetrahydro-te is produced by the enzymic oxida-of "active formaldehyde" (Osborn and ennekens, '57). Similarly, only N^5,N^{10}-thenyl tetrahydrofolate can be reduced mically by borohydride to "active form-ehyde" (Huennekens and Osborn, '58).

b) The *pH* optimum curve for the mical synthesis of "active formalde-hyde" from HCHO and tetrahydrofolate reveals the obligatory participation in the reaction of two prototropic groups, having pK_a values of 3.0 and 5.4 (Osborn *et al.*, unpublished data). These values correspond closely to the pK_a values of the N^{10} and N^5 atoms, respectively, of tetrahydrofolate.

(c) The equilibrium constant for reaction (19) (fig. 14), $\sim 10^4$ at *p*H 7 (Huennekens and Osborn, '58; Blakley, '58), is much greater than would be anticipated for a single linkage between HCHO and one nitrogen atom. Adduct formation of the type $CH_2(OH)_2 + R_2NH \rightleftharpoons HOCH_2—NR$ usually has a *K* value of only 10^1–10^2.

A mechanism for the chemical catalysis of reaction (19) must take into account the fact that synthesis is maximal at *pH* 4.2, where the N^5 atom is charged and the N^{10} atom is uncharged. In the mechanism pictured in figure 15, HCHO first forms an adduct with the uncharged N^{10} atom,[5] and this is followed by a concerted displacement of electrons leading to the expulsion of OH^- from the C_1 group and H^+ from the N^5 atom; bond formation between the methylene carbonium ion and the nucleophilic N^5 atom then follows. If a similar mechanism were operative during en-

[5] In the Mannich reaction, and similar cases where HCHO forms adducts with amines, the uncharged amine is assumed to be the reactive species. The weaker basicity of the N-10 position as compared to the N-5 position, accounts for the fact that formylation of tetrahydrofolate with concentrated formic acid leads first to the formation of N^{10}-formyl tetrahydrofolate and then to N^5,N^{10}-methenyl tetrahydrofolate. It is of interest, too, that enzymic synthesis and deacylation of "active formate" [reactions (1) and (11)] involve only N^{10}-formyl tetrahydrofolate.

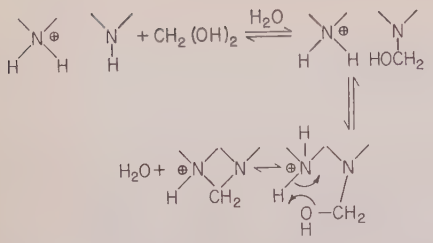


Fig. 15 Tentative mechanism of "active formaldehyde" formation.

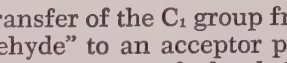
zymic catalysis of reaction (19) at neutral *p*H, the initial condensation of HCHO might occur at either position, since both nitrogen atoms would be uncharged. Removal of the —OH group, as a prerequisite to ring closure, is accomplished by the formaldehyde-activating enzyme at neutral *p*H; this may be contrasted to the chemical synthesis where this step is best achieved in the presence of acid. Therefore, the *p*H dependence of carbonium ion formation in the methylene group may also contribute to the *p*H optimum curve for chemical synthesis of N^5,N^{10}-methylene tetrahydrofolate.

The same mechanism also accounts for the effect of *p*H on the stability of N^5,N^{10}-methylene tetrahydrofolate toward HCHO-trapping agents such as H_2NOH. In an acidic medium, the sequence in figure 15 is readily reversed, especially when the equilibria are shifted through the removal of free HCHO. Conversely, in basic solutions, N^5,N^{10}-methylene tetrahydrofolate has the structure shown at the top of figure 16,

and there is little tendency for the requis polarization of either C—N linkage occur.

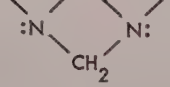
Transfer of formaldehyde from N^5,N^1 methylene tetrahydrofolate to acceptors

The transfer of the C_1 group from "acti formaldehyde" to an acceptor probably volves the opening of the bridge stru ture to form a transient intermedia



as shown by the alternate pathways reactions (20) and (21)(fig. 16). In eith reaction, the polarized $C^{\delta+}$—$N^{\delta-}$ bond attacked by H^+ and OH^-, or H^+ and R^-.

In the biosynthesis of serine from N^5,N methylene tetrahydrofolate and glyci only a simple transfer of the C_1 group involved. In the synthesis of methioni thymidylic acid, and choline, transfer the C_1 unit from N^5,N^{10}-methylene tet hydrofolate to an acceptor must be follow by a reduction of the C_1 unit to the met level. The reductive step, possibly m diated by a coenzyme form of vitamin could occur by the pathway in reacti (22) or that in (23) (fig. 17). Reacti (23), postulated in studies of dimeth glycine oxidation (Mackenzie and Fris '58), is of special interest, since it impl the existence of a new C_1 structure at oxidation level of formaldehyde. The c responding structure of "active formal



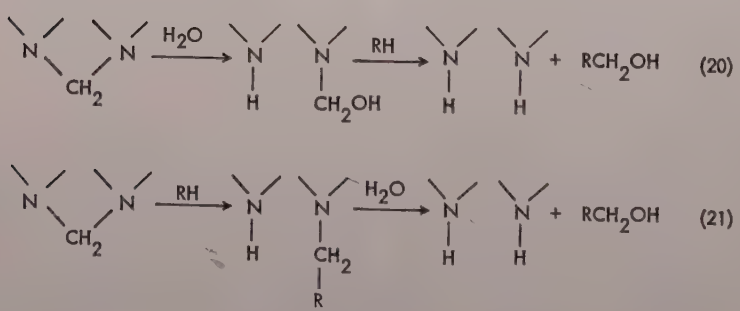
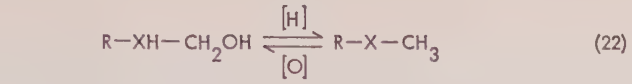


Figure 16



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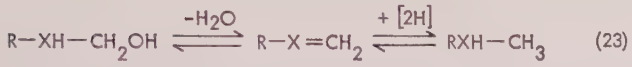

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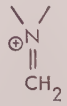

Figure 17

e" would be written as shown at the tom of figure 17, with the methylene up doubly bonded to only one nitrogen n.

DISCUSSION

he mechanisms suggested in this paper the various reactions involving the *de o* synthesis and hydrolysis of C—C or N bonds involving C_1 units at the level HCOOH or HCHO (summarized in table have certain characteristics in com- n. A prototype for the synthetic reac- s is provided by the base-catalyzed isen condensation or aldol condensation C—C bonds [reaction (24), fig. 18] or, parably, the Mannich reaction for N bonds [reactions (25a–c), fig. 18]. trong base, such as sodium ethoxide, is required to promote carbanion formation in one of the reacting molecules; polarization of a C=O bond produces the carbonium ion in the other participant. For convenience in the ensuing discussion, the reactants containing the carbonium ion and the carbanion, respectively, will be designated as the donor and acceptor.

As shown in table 2, the various reactions where HCHO acts as the donor can be separated into three categories, depending on the requirement for coenzymes: (a) neither HCHO nor the acceptor (tetrahydrofolate or, *inter alia*, pyruvate or ketovaline) requires activation, i.e., union with a coenzyme; (b) HCHO requires activation to N^5,N^{10}-methenyl tetrahydrofolate but the acceptor (e.g., the precursors of thymidylate and methionine) does not; (c) both

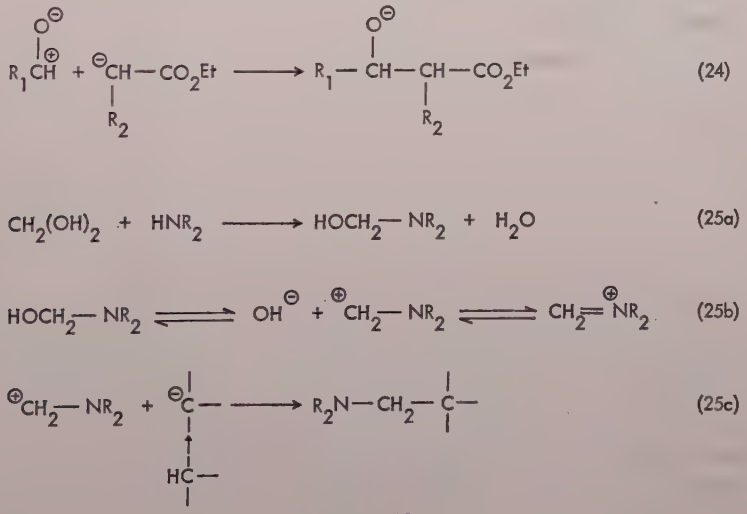

Figure 18

TABLE 1

Reactions types involving C_1 units

	Reaction	Chemical catalysis	Enzymic catalysis
Reactions of HCOOH			
(1) Amide formation	Formate + tetrahydrofolate → N^{10}-formyl tetrahydrofolate + H_2O	H^+	HCOOH-activating enzyme; requires ATP[b]
(2) Orthoamide formation	N^{10}-Formyl tetrahydrofolate + H^+ → (N^5,N^{10}-methenyl tetrahydrofolate)$^+$ + H_2O[a]	H^+	Cyclohydrolase
(3) Orthoamide formation	N^5-Formyl tetrahydrofolate + H^+ → (N^5,N^{10}-methenyl tetrahydrofolate)$^+$ + H_2O	H^+	Folinic isomerase; requires ATP[b]
(4) Orthoamide formation	N^5-Formimino tetrahydrofolate + $2H^+$ → (N^5,N^{10}-methenyl tetrahydrofolate)$^+$ + NH_4^+	Unknown	Cyclodeaminase
(5) Amide hydrolysis	Reversal of (1)	H^+	N^{10}-Formyl tetrahydrofolate deacylase
(6) Orthoamide hydrolysis	Reversal of (2)	OH^-	Cyclohydrolase
(7) Orthoamide hydrolysis	Reversal of (3)	OH^-	Unknown
Reactions of HCHO			
(8) Aminohemiacetal formation and hydrolysis	Formaldehyde + tetrahydrofolate ⇌ N^5- or N^{10}-hydroxymethyl tetrahydrofolate + H_2O	H^+	HCHO-activating enzyme
(9) Aminoacetal formation and hydrolysis	N^5- or N^{10}-Hydroxymethyl tetrahydrofolate ⇌ N^5,N^{10}-methylene tetrahydrofolate + H_2O	H^+	HCHO-activating enzyme

[a] Stimulated by phosphate.
[b] Inhibited by phosphate.

TABLE 2

Coenzyme requirements for reactions involving formaldehyde

Type	"Donor"	Coenzyme	"Acceptor"	Coenzyme	Product
A	HCHO	None	Tetrahydrofolate	None	"Active formaldehyde"
	HCHO	None	Pyruvate	None	γ-Hydroxy-α-ketobutyrate
	HCHO	None	Ketovaline	None	Ketopantoate
B	HCHO	Tetrahydrofolate	Homocysteine	None	*S*-Hydroxymethyl homocysteine (?)
	HCHO	Tetrahydrofolate	Deoxyuridylate	None	5-Hydroxymethyl deoxyuridylate
C	HCHO	Tetrahydrofolate	Glycine	Pyridoxal phosphate	Serine

nor and acceptor require activation with coenzyme (HCHO with tetrahydrofolate d glycine with pyridoxal phosphate). esumably, adduct formation between a enzyme and the donor or acceptor is cessary to assist the enzyme in accom- shing the required bond polarization ding to the carbonium ion or carban- n. For example, in serine biosynthesis,)—CH_2—NR_2 (i.e., the transient form "active formaldehyde") is more suscep- le to carbonium ion formation than)—CH_2—OH, whereas Schiff's base for- tion between glycine and pyridoxal phos- ate withdraws electrons from the α-car- n atom and permits carbanion forma- n at this site.

Reactions with HCOOH as the donor ay also be examined in terms of potential rbonium ion formation. Creation of a rbonium ion in HCOOH is more difficult, wever, than in HCHO, owing, in part, to sonance stabilization of the carboxylate n. For this reason, chemical formylation the N-10 position of folic acid or tetra- drofolate occurs only with concentrated rmic acid. Acetic anhydride catalyzes is reaction, suggesting that the attacking ecies is actually a mixed anhydride of rmic and acetic acids. Similarly, the cile formation of the orthoamide (N^5, ^{0}-methenyl tetrahydrofolate) by acid eatment of N^5- or N^{10}-formyl tetrahydro- ate [reaction (12), fig. 10], by cyclo- drolase acting upon N^{10}-formyl tetra- drofolate [reaction (9), fig. 6], by cyclo- aminase upon N^5-formimino tetrahydro- ate [reaction (10), fig. 7], or by folinic merase upon N^5-formyl tetrahydrofolate eaction (8), fig. 6], may be attributed to e preexistence of the formate as an nide. In the enzymic synthesis of N^{10}- rmyl tetrahydrofolate [reaction (1), fig. however, formylation of the acceptor curs via free formate, but the acceptor is st activated by phosphorylation, as out- ed in figure 4.

It should be emphasized that the func- n of the enzyme in the reactions just cussed is twofold: (a) to fix the donor d acceptor in suitably adjacent positions; d (b) to provide, via electropositive and ctronegative centers at the "active site," neans for enhancing the desired polariza- ns leading to the formation of the car- bonium ion and carbanion. It is not neces- sary for the C—H bond in the acceptor to be formally broken, only that it be polar- ized with the aid of a negative center on the enzyme to the extent that a potential carbanion is created. Likewise, a positive center on the enzyme could enhance polar- ization of the C=O group in the donor. The desired C—C (or C—N) bond would then be formed by the smooth, concerted movement of electrons.

ACKNOWLEDGMENT

We are indebted to Drs. P. T. Talbert, L. L. Ingraham and H. R. Mahler for help- ful discussions of the mechanisms in this paper.

OPEN DISCUSSION

HARTMAN[6]: We have carried out some preliminary experiments concerning the nonenzymic interconversion of N^{10}-formyl and N^5,N^{10}-anhydroformyl tetrahydrofolic acid. Phosphate catalyzes this reaction quite effectively, but in the presence of Tris chloride buffer the reaction also takes place at a markedly increased rate com- pared to that in the presence of maleate buffer. At *pH* 7.4 the times for half reac- tion are 28 minutes in maleate solution, 5.1 minutes in Tris chloride, and 2.3 minutes in phosphate solution. So if we are going to postulate a mechanism for this reaction, the specific effect of these various anions should be accounted for.

I wonder if any of the chemists here would care to comment on what would seem to be a rather unusual reaction in the course of the activation of formate. The second step of this process, according to Dr. Huennekens's formulation, is in ef- fect a transamidation from a phosphoram- idate compound to the carboxyl group of formic acid. This is presumably a nucleo- philic attack on the carboxyl group by a nitrogen atom that is substituted by the electron-attracting phosphate group. This should tend to make it a poor nucleophilic agent. I think a similar situation exists in the reaction that Dr. Gunsalus proposed for the activation of succinic acid.

BRESLOW[7]: I just want to elaborate on some things that Dr. Koshland has men-

[6] S. C. Hartman, Massachusetts Institute of Technology.

[7] Ronald Breslow, Columbia University.

tioned. What is involved, as you point out, clearly is not the attack of the phosphorylated nitrogen on the carboxyl group. What we have to do is to transfer phosphate from the nitrogen onto the formate, and that releases the nitrogen and generates formyl phosphate in the vicinity, which would then react with the nitrogen to formylate it. The second step might be very fast.

BUCHANAN[8]: Dr. Huennekens, have you carried out experiments on the arsenolysis of the N^{10}-formyl tetrahydrofolic acid? I think an experiment of this type would be very crucial to the role of phosphoryl tetrahydrofolic acid you are proposing in the synthesis of formyl tetrahydrofolic acid from ATP, formate, and tetrahydrofolic acid. I would suspect that arsenate could replace phosphate in the reverse reaction and that the presumed intermediate N^{10}-arsenate tetrahydrofolic acid would be rather unstable and would decompose. If that were the case, I would expect that adenosine diphosphate would not be necessary as an acceptor of arsenate in the formation of free tetrahydrofolic acid, since it would be needed as an acceptor of the phosphate of the corresponding phosphoryl compound and since the latter substance would be presumably much more stable than the arsenate analog.

HUENNEKENS: Do you mean studying the enzymic action in reverse?

BUCHANAN: In reverse. I think this would be a critical experiment for the postulation of the N^{10}-phosphoryl tetrahydrofolic acid. Also, have you done any oxygen transfer experiments?

HUENNEKENS: No, we have not carried out O^{18} experiments. [Note added in proof: Dr. Buchanan's interesting suggestion has now been tested experimentally. In reaction (3), arsenate does not stimulate the disappearance of N^{10}-formyl tetrahydrofolate in the presence or absence of ADP.]

SAKAMI[9]: There is another possibility of active formate formation, and I wonder whether Dr. Huennekens has considered it. Some time ago Strittmatter and Ball purified a formaldehyde dehydrogenase that required glutathione as a coenzyme. They postulated the formation of *S*-formyl glutathione as an intermediate of the formation of formate from formaldehyde. I wonder whether in animal tissues the conversi of formaldehyde to active formate proce to a major extent by way of the formati of *S*-formyl glutathione and transfer of *S*-formyl group to tetrahydrofolic acid.

There is a second and lesser possibili Dr. Mackenzie has reported that thiaz dine carboxylic acid, which is formed i spontaneous reaction between formal hyde and cysteine, is oxidized by mi chondria. This may involve *S*-formyl c teine formation, and perhaps we may ha a transfer of the *S*-formyl group to tet hydrofolic acid. Do you care to comm on this?

HUENNEKENS: Yes, we have been aw of these reactions. The first one, i.e., Strittmatter and Ball enzyme, involves duct formation between glutathione a formaldehyde followed by oxidation formyl glutathione. The latter compou was sufficiently labile to regenerate "coenzyme," glutathione, and yield f mate. In a sense, this sequence is ana gous to the reactions catalyzed by droxymethyltetrahydrofolic dehydrogen where HCHO is condensed with the co zyme, tetrahydrofolate, and then oxidat occurs at the expense of TPN instead DPN. Finally, hydrolysis of "active f mate" into tetrahydrofolate and form completes the sequence. We have fou the Strittmatter and Ball enzyme in so of our dehydrogenase preparations esp ally in the crude stages, but this is remov by further fractionation.

With regard to the second system t involves formaldehyde and cysteine c densing to give thiazolidine carboxylic a the oxidation occurs in the ring to g thiazoline carboxylic acid. Isn't it true t this product does not come apart too re ily to yield formate and regenerate cysteine?

SAKAMI: I believe that Mackenzie tablished the formation of the *N*-for compound but did not exclude the for tion of an *S*-formyl derivative as well.

Another question that I would like raise concerns some work carried out my laboratory by Dr. Anderson, as a m

[8] J. M. Buchanan, Massachusetts Institut Technology.

[9] Warwick Sakami, Western Reserve Univer

student. He incubated formaldehyde, ahydrofolic acid, and glycine with a on liver extract, and found that amino- in increased the formation of serine decreased the formation of N^{10}-formyl ahydrofolic acid. This suggests that nopterin blocks the interconversion of ve formate and active formaldehyde.

UENNEKENS: We have not observed inhibition of the hydroxymethyltetra- rofolic dehydrogenase by aminopterin. should like to raise a question in the e that somebody here can supply an wer. We have been very much inter- d in the formate-pyruvate exchange re- on, which has been reported to be de- dent on tetrahydrofolate. Does any- here have any current information on particular problem?

AKAMI: Dr. Chin, in Dr. Krampitz's ratory, has worked on this problem. did find that tetrahydrofolic acid had e effect on the phosphoroclastic reac- , but I do not know whether he would willing to call it a cofactor of this ess. Tetrahydrofolic acid is a reducing nt, and it may in some processes act his way.

UCHANAN: Do you think if you carried your synthesis of the N^{10}-formyl tetra- rofolic acid with all the agents present might get a kind of concerted or si- taneous reaction that would avoid this er difficult intermediate, the N^{10}-phos- yl tetrahydrofolic acid? Perhaps we ht look at this compound as being a of side product, which is utilized but ly not necessary for the reaction when carried out in the over-all process; with ATP, formate, and tetrahydrofolic all present at the beginning of the in- ation.

UENNEKENS: This question recalls dis- ions on the role of the acyl adenylates ne activation of carboxylic acids. But ther the proposed phosphorylated tetra- rofolate, like acyl adenylates, occurs mall amounts as a free intermediate or ther it is formed only transiently as nzyme-bound complex is still in doubt.

UCHANAN: I do not mean that. I n do you think it even exists, even he enzyme site.

UENNEKENS: The point I was making simply that, in many of these com- plex "activation" reactions, we can assume that the three reactants are so arranged on the enzyme that partial bonds are formed between individual reactants dur- ing different phases of the reaction. In a formal way, then, we can consider the formation of "intermediates" in the reac- tion, although they are enzyme bound. The evidence in the formate-activating system would favor the implication of phosphoryl tetrahydrofolate as an inter- mediate. The failure of hydroxylamine to inhibit the over-all reaction would argue against formyl phosphate as an intermedi- ate, as proposed a moment ago by Dr. Breslow. We simply have to try to derive a reasonable mechanism to account for the experimental facts.

SAKAMI: We have centered our atten- tion on folic acid involvement in formate and formaldehyde utilization. To balance this consideration of one-carbon metabo- lism, I would like to point out that there are many reactions of formaldehyde and processes in which formate is formed that appear to have nothing to do with folic acid. Some of these processes are the formation of α-keto-γ-hydroxybutyric acid from formaldehyde and pyruvate, the for- mation of erythrulose phosphate from di- hydroxyacetone phosphate and formalde- hyde, and the formation of ketopantoic acid. There are a number of one-carbon processes in which, as far as we know, tetrahydrofolic acid is not involved.

METZLER[10]: When we talk about active acetate or active formate we are talking about something that is thermodynamically unstable with respect to the products of a transfer reaction. This active formalde- hyde has been deactivated with respect to thermodynamic reactivity. Perhaps we ought to be calling this bound formalde- hyde, rather than active formaldehyde.

HUENNEKENS: I would not entirely agree with that argument, although it has considerable merit. I think the term "ac- tive" has been used in the past simply to indicate an adduct between a mobile metabolic group and a coenzyme.

SAKAMI: Sometimes the term "active" refers largely to the fact that it is a point of active research interest.

[10] D. E. Metzler, Iowa State College.

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ıbstrate Specificity of Chain Propagation Steps Saccharide Synthesis

SHLOMO HESTRIN[1]
Hebrew University, Jerusalem, Israel

Some of the conventional partitions that ve been used to classify enzymes into egories are falling. The discovery of esteratic function of proteinases pro- es a particularly striking illustration of s trend. Other examples are found ıong the carbohydrases, which were once idly divided into hydrolases and trans- ases. There are still a few carbo- drases for which no function of trans- to a nonwater acceptor is known; number of carbohydrases also exist ıt have transfer but no detectable drolytic activity. The large majority of rbohydrases, however, are apparently le to catalyze glycosyl transfer to a var- y of alternate acceptors and water as ll. Similarly, it was formerly thought to useful to subclassify carbohydrases of e transferring type as catalysts of forma- n of (1) oligosaccharides and (2) mac- molecules. In a number of cases, a given nor system can serve with one protein stem for oligosaccharide production but, th a different protein system, lead to acromolecule production. In some of ese systems, however, the end products the reaction include coexistent macro- olecular as well as oligosaccharidic pol- ıer species. Thus rigid separation of en- me types according to the polymerization gree of the end product of reaction might misleading.

Donor molecules participating in en- mically promoted reactions of chain opagation all conform to a common ructural pattern. They contain a glycose sidue that bears a substituent (X group) the anomeric oxygen atom. In reaction ey are cleaved at this site, the glycose sidue being transferred to an acceptor e from which X acquires a hydrogen om and thereby is released into the solu- n. An important function of X is its ability to maintain the donor system at an energy level in which the transfer to the growing chain is a thermodynamically favored outcome in the biological situation (see Kalckar, '54).

Donor systems have been classified according to the nature of X. As is now apparent, X can be orthophosphate (as in α-glucose 1-phosphate), derivatives thereof (such as uridine phosphophosphate in uridinediphosphate glucose), glycosyl (as in glycosylglycosides; e.g., sucrose), or glycose (as in glycosidoglycoses; e.g., maltose). One or several forms of X can be consistent with donor activity in any given enzyme species. The variation in this respect, as between different transferring enzymes, is wide indeed (for reviews of this aspect, see Hehre, '51; Barker and Bourne, '53; Bacon, '53; Kalckar, '54; Edelman, '56; Hestrin, '58).

Still another key to a classification of donor systems might be their possession or lack of a carbinol group that can function as a potential or explicit acceptor site (A site) for the transferred glycose group. In the simplest case, the activity of the A site is relatively insensitive to the size or nature of the substituent on the anomeric oxygen. Then transfer can occur in absence of any added "primer," and the end product of the transfer reactions is a homologous polymer series. Frequently, however, the activity of the A site is markedly affected by the nature and bulk of the substituent on the anomeric oxygen. In a particularly important group of reactions conforming to this pattern, X abolishes the activity at A. These are the reactions in which transfer can occur only if exogenous acceptor ("primer") is intro-

[1] Visiting Scientist, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.

duced into the system. Where hexose on the anomeric oxygen suppresses as X the activity at A, the reaction becomes rigidly restricted to disaccharide formation. Where, on the other hand, transfer, with the attendant increase of the bulk of the substituent on the anomeric oxygen, enhances the activity at A and especially where the transfer multiplies the number of active A sites, polyrepetition of transfer is favored, and giant macromolecules can emerge. Depending on the particular form assumed by the *A activity—polymerization degree* function—they emerge either side by side with the oligosaccharides or practically exclude the latter in the reaction product. I shall not attempt here to survey all the carbohydrases from this point of view. The principles involved can readily be derived from the particular cases to be discussed.

Knowledge of maltose phosphorylase (Fitting and Doudoroff, '52) affords an instructive example of an A-site activity that responds to changes of structure at atoms several carbon units removed. The reaction has been formulated as:

$$\beta\text{-glucosyl 1-phosphate} + \text{glucose} \rightleftharpoons \text{maltose} + \text{orthophosphate} \qquad (1)$$

The A site here is in the C-4 carbinol position in glucose. With a free reducing function at C-1 in the acceptor system (free glucose), the A site manifests high activity, as witness production of maltose in this system. However, if the free reducing function is blocked by a substituent, e.g., by a phosphoryl group as in the donor (β-glucosyl 1-phosphate) or by a glycose group as in the disaccharidic product (maltose) of the observed transfer reaction, the A site becomes inactive, as witness the apparent nonappearance in this reaction mixture of any amylose homologs of degree of polymerization > 2 or of chains terminated by a phosphate group.

A rather different situation prevails where maltose is acted upon by the bacterial amylomaltase (Barker and Bourne, '52). Here the reaction is:

$$\text{Maltose} + (\text{glucose})_n \rightleftharpoons (\text{glucose})_{n+1} + \text{glucose} \qquad (2)$$

Here $(\text{glucose})_n$ represents the homologous amylose series with $n \geqslant 1$. In this case, too, C-4 in the free glucose is an active A site. In contradistinction to the situa encountered with maltose phosphoryl however, substitution of C-1 in glucose glycose as in maltose fails to suppress activity of A. Increase in the size of glycose on C-1 apparently neither fa nor suppresses the activity of A to marked degree. Thus, in this system continuous series of amylose homolog generated, but the major fraction of product is in the oligosaccharide range tends to be so as long as glucose is in system in sufficient concentration to c pete at its A site for the transferred cosyl moieties.

A higher degree of complexity is countered where donors of different cose moieties can participate in a trans system, e.g., in the action of *Baci macerans* amylase. Both single gluc as well as polyglucosyl groups can be tra ferred by this enzyme; moreover, the do can be either a linear amylose homolog a cycloamylose. French ('57) aptly dicted of this system that it will conti "to serve, delight, teach and intrigue carbohydrate chemist for many years come." In one respect at least, howe this system may be relatively simple. with amylomaltase, acceptor activity of A site with Schardinger amylase seems be remarkably tolerant of variation of b and structure of the substituent on anomeric oxygen. Another disproporti ing enzyme, the D enzyme, likewise tra fers whole $(\text{glucosyl})_n$ residues fr donors of type $(\text{glucosyl})_{n+x}$, where n have a variety of values and C-4 carbino free glucose is a favored A site. Subst tion on the anomeric oxygen of gluc markedly lowers the activity of the A in this system (Peat *et al.*, '57). Thus such a case, the heterogeneity of the product of the reaction stems partly fr the presence of competing acceptors partly from the participation of compet donors, presenting to the enzyme a vari of transferrable groupings.

Specificity rules governing acceptor tivity are modified remarkably in individ phosphorylase systems. In this group enzymes, A-site activity in the donor b ing phosphate on the anomeric oxygen always nil, but so is the activity of A in the free hexose. If glucose is put on

meric carbon site as in maltose, again e is no activity at A. But if maltose is on the anomeric carbon, the A site ws some acceptor activity when the lyst is potato phosphorylase. If the size ne aglycon is increased by putting amyl- homologs on the anomeric carbon, the ctivity increases dramatically through- a critical range of amylose size. How- r, further increase of the amylose size rs the acceptor activity of the A site to uch smaller extent. Whelan and Bailey 4) have presented a particularly elegant lysis of the consequences of this situa- . They showed that the priming activ- of maltotriose in comparison to that of totetraose was of the order of $1:10^4$. a system primed by maltotriose, we ld expect accordingly that a few mole- es of maltotetraose would effectively pete as acceptors with a relatively large nber of molecules of maltotriose and ld therefore be rapidly converted into topentaose. The latter would then, in , compete effectively with the abun- t maltotriose. By repetition of this e, long chains would tend to be formed a process conforming in appearance single-chain growth mechanism lead- preponderantly to a highly polymerized duct would be observed. If, however, icient maltotetraose or higher homolog e added to a system as primer, the true tichain nature of the reaction might expected to become evident, and the rage polymerization degree of the end duct would be correspondingly lowered. se expectations were fully confirmed. Iuscle phosphorylase provides a par- larly instructive example of a system which rigid requirements for aglycon cture must be met if activity of A is to ur. Apparently, an acceptor for the scle system is not really good unless it in the aglycon a group with the bulk branched kind of structure like the e of glycogen itself (Cori *et al.*, '45). , even if there is such a core in the ycon, there are still remarkable local uirements to be satisfied before chain gation can begin. A good example of feature could be derived from an ex- ment that I was able to carry out dur- a visit to Professor Cori's laboratory ne years ago. The primer activity mani- fested toward the muscle enzyme by the phosphorylase limit-dextrin was compared with the β-amylase limit-dextrin of glycogen. We know now, from the later work of Cori and Larner ('51), that outer stubs in the phosphorylase limit-dextrin of glycogen have the following arrangement.

where E represents an amylose chain of about five glucose residues, attached to a glucose unit (C) bearing a single glucosyl residue (D) at C-6 and glycogen core (R) on the anomeric oxygen. It can be seen that in this structure β-amylase will act on the molecule by removing two maltose residues from the amylose chain. Limit-dextrin formed from glycogen by muscle phosphorylase is a good acceptor of glucosyl residue transferred by muscle phosphorylase, but the limit-dextrin generated from glycogen by β-amylase was not (Hestrin, '49). Thus the remarkable result is that, for the A site of the glucosyl residue D to act as an acceptor, there must be a glucose unit (C) on the anomeric oxygen of this residue that carries a sufficient substituent on both its carbons 1 and 4.

Information concerning primer relations of enzymes that catalyze polymer formation from uridinediphosphate glycoses acting as donor systems is still rather limited and may not as yet permit similar analysis. Several important reactions in this group are listed in table 1. An unexpected property of several of those systems is their dependence on an activator. In every case the latter bears some resemblance to the actually transferred glycosyl group. In the glycogen-generating system, glucose 6-phosphate is such an activator (Leloir *et al.*, '59). A variety of simple glycosides similarly activate the callose-generating enzyme (Feingold *et al.*, '58). *N*-Acetylglucosamine activated a particulate, though not the soluble form, of the chitin-generating enzyme from *Neurospora crassa* (Glaser and Brown, '57). It may be noteworthy also in this connection that *N*-acetylglucosamine 1-phosphate is an activator of hyaluronic acid synthesis in a system consisting of an ATP-fortified sonicate of streptococcus and an appropriate "uridinediphosphate glycose" donor system (uridinedi-

TABLE 1

Some reactions involving as the donor system a uridinediphosphate glycose and leading to a homopolymeric product

Polymeric product		Glycose moiety in donor	Primer[a]	Enzyme source	Reference
Name	Kind of interglycosidic linkage formed				
Glycogen	α-1, 4	Glucose	Glycogen	Rat liver	Leloir *et al.*, '59
Cellulose	β-1, 4	Glucose	Cellodextrins	*Acetobacter xylinum*	Glaser, '58
Chitin	β-1, 4	*N*-Acetyl glucosamine	Chitodextrins	*Neurospora crassa*	Glaser and Brown, '57
Callose	β-1, 3	Glycose	—[b]	*Phaseolus* seedlings	Feingold et al., '58

[a] Added "primer" markedly augmented the velocity of the observed polymerization. That there is incorporation of primer into the finished polymeric product is assumed but for the most part has yet to be proved.

[b] Although the reaction catalyzed by this system was stimulated by a large variety of added glycoses, it has been suggested that the latter are probably not primers in the usual sense, i.e., that they are not incorporated into the finished polymer.

phosphate glucuronic acid plus uridine phosphate *N*-acetylglucosamine) (D man *et al.*, '58). The possibility that s activations may involve activity on part of the added compounds has still to tested by an unequivocal method. If added compound has itself a very low ceptor activity and gives rise during action to a small amount of compou with high A-site activity, incorporation the added compound into the formed ymer might easily escape detection, would the added compound in such a c markedly depress the polymerization gree of the polymer formed. An interest alternative to explain the observed effe is suggested by Koshland's theory of duced fit." According to this view (Ko land, '59), activators of an enzyme th selves might be either substrates or a logs thereof capable of combining with protein surface near its active site modifying its conformation so as to ren the site an effective catalyst.

Transfer reactions involving an a sylfructoside (notably sucrose) as do system comprise several interesting amples of the relation of activity to st ture and bulk of a substituent on anomeric oxygen. A further study of tr fructosylation from sucrose as catalyzed the classical yeast invertase (see, Ba '53; Edelman, '56) from this point of v might be of particular interest. In the y invertase system, the major A site a from water itself is the primary carb group at C-6 in a nonreducing term fructose unit. The polymerization obse with yeast invertase does not proceed detectable extent beyond the oligosacc ide level. In this respect it contr sharply with the analogous transfructos tion reaction catalyzed by levansucr An explanation that can be considere that, in the invertase system, the activit the A site fails in the pertinent rang increase with the bulk of the substit on the anomeric oxygen of the term fructose residue. The concentration polymer at the successive levels of

erization degree (n) therefore shows a arp decrease with n at any selected time. the transfructosylation catalyzed by *robacter levanicum* levansucrase with crose as the exclusive added substrate, gosaccharide represents an appreciable oportion of the reaction end product only a relatively high concentration of sucrose used. At substrate concentrations in the nge of half-saturation of the enzyme, wever, almost the entire mass of the ansferred fructose other than that part verted to water is recovered as macro-olecular levan with a molecular weight the order of 10^7–10^8 (Hestrin *et al.*, '56; ingold *et al.*, '56). Two circumstances obably participate critically in this re-lt. One of them is that the chain elonga-on is accompanied here by branching; a result, the number of A sites in the owing molecule of polymer increases th its polymerization degree (n). In ldition, it could be postulated that A sites the growing large polymers have activity eatly exceeding that of similar A sites oligosaccharides in the low polymeriza-on degree range. If the reaction is con-ived as proceeding exclusively on added crose in the absence of extraneous ac-ptor, this concept would imply that, as ain elongation proceeds on a sucrose-rminated polymer,a range of n is eventu-ly entered in which acceptor activity at increases rapidly with n and a high tivity of A thus attained is subsequently tained on further increase of n. To test is hypothesis, we measured acceptor ac-vity with levansucrase as manifested by ccessive members of a homologous series reducing levulans obtained from the rtial acid hydrolyzate of macromolecular van by appropriate fractionation (D. S. ingold, G. Avigad, and S. Hestrin, un-blished experiments). A dramatic in-ease in A activity with n throughout a itical range of n was, in fact, found. The west two members of this series (levan-ose and levantriose) showed a very low ceptor activity. However, in the range = 3 to 6 or 8, activity increased roughly by a factor of at least 2 at each successive level of n. A specific relation between the enzyme and the grouping on the anomeric oxygen of the A-bearing fructose unit has to be assumed in this connection, since bulk alone does not produce this effect. Thus we found that inulins of similar and higher n than the tested levulans were, quite unlike the last named, practically devoid of acceptor activity.

Degraded levan added to the reaction mixture may cause an increase in the rate of the transfructosylation reaction, but the enhanced competition for transferred fructose exerts an inhibitory effect on fructose formation from sucrose. Peaud-Lenoel ('57) showed that the effect of the added levulan is noncompetitive with respect to sucrose. This leads to the suggestion that the binding site for acceptor levulan in levansucrase is distinct from the site of the binding of donor sucrose.

The first step in the reaction series leading from sucrose to levan commands particular interest. If, as seems probable, chain growth proceeds stepwise by successive single additions of fructose to the acceptor and if the primary acceptor is sucrose, the intermediate first formed from sucrose in the course of levan formation might be either 1^F-fructosylsucrose (1-kestose) or 6^F-fructosylsucrose (6-kestose) or both. One or both of these substances might be expected not only to be formed but also to be capable of further interactions with sucrose in the presence of levansucrase and lead eventually to levan production. Formation of both 1^F- and 6^F-fructosylsucrose as well as other oligosaccharides formed by fructose transfer to carbinol sites does indeed attend levan production from sucrose. It should be noted, however, that an accumulation of an oligosaccharide during levan production does not by itself constitute proof that this saccharide is an intermediate rather than a mere by-product of levan production.

Kohanyi and Dedonder ('51) first observed the formation of trisaccharide from sucrose in their study of the *Bacillus sub-*

tilis levansucrase system. They assumed but did not rigidly prove that the trisaccharide was 6^F-fructosylsucrose. Working with the *A. levanicum* enzyme at suitably high sucrose concentration, we likewise noted an abundant formation of trisaccharide but discovered to our surprise that this product was largely 1^F-fructosylsucrose; the isomer 6^F-fructosylsucrose was present only in trace amounts. A similar situation was noted in cultures of a levan-forming *Corynebacter* sp. In view of these results the possibility that the major trisaccharide formed from sucrose by the *B. subtilis* system is 1^F- rather than 6^F-fructosylsucrose may be worth reexamination.

Attempts to demonstrate a build-up of high-molecular levan from any of the oligosaccharides occurring in a sucrose-levansucrase digest have not so far succeeded. Several single successive additions of fructose to these products proved possible. Starting with 1^F-fructosylsucrose, we have synthesized chains containing as many as five fructose units on a glucose terminal stepwise by interaction of the acceptor with sucrose in the presence of levansucrase. However, increase of priming activity with chain length was still not observed in this oligosaccharide series; rather, there was a drop in acceptor activity with the increase of n. Addition of the oligosaccharide to sucrose failed to exert any large effect on either the amount or turbidity of high-molecular levan formed. Of course, there is still a possibility that, at some eventually reached polymerization degree, a condition is achieved in which the priming activity of the glucose-terminated fructose chains begins to rise with n and enables rapid build-up of macromolecular levan by further transfructosylation. There is no proof of this. The fructose chains on glucose in the series built up from 1^F-fructosylsucrose conform in chromatographic mobility to a polymer homologous series; the plot of the mobility function log α^1 against n in this series gave a straight line whose slope was equal to that of the inulin series. Thus it is likely but not certain that the fructose units in this series of oligosaccharides a connected by a linkage of the type 2 → i.e., as in inulin and not typically as levan. If these compounds are indeed ulins rather than levans, it would be prop to doubt that they are intermediates levan production. They might instead products formed by levansucrase in a pr ess of oligorepetitive transfer that is subj to a low polymerization ceiling and par lels but does not underly that avalanch like process of polyrepetitive transfer which the giant molecules of levan are p duced.

If sucrose is not the primary accept in levan production, the primary accept must be an as yet unknown component the reaction system. It might be a su stance formed by an interaction betwe enzyme and sucrose at a site other th carbinol. The primer, it should be stresse need not necessarily be a fructose polym Since it is known that levansucrase d places the glucose group from sucrose, t possibility that the initial primer is a fr tosyl protein formed from sucrose by placement of a glucosyl group by prote deserves consideration. The levan mo cule is so large that even a large init primer, such as a protein built into the fi product, would not seriously lower the fr tose content of the latter. Moreover, ev a small number of primer molecules co well go a very long way in terms of fr tose polymerization before symptoms deficiency as to primer in the polymeri tion system would become manifest.

A discussion of glucose transfer fr sucrose by the action of dextransucra could proceed along almost the same li as have been followed in reference levansucrase. In the dextransucrase s tem, too, there is multiplication of A si incidental to the polymerization. As in t case of levansucrase, the finding that ceptor activity of oligosaccharidic dextra is low in comparison to that of intern diary-range dextrans implies that A activ in the dextran series increases dramatica with n within an as yet undefined criti

ıge (Hehre, '53; Koepsell *et al.*, '53; uchiya *et al.*, '53; Stringer and Tsuchiya, 3).

Donor systems participating in enzymi-lly catalyzed reactions of chain propaga-ın appear to be specifically suited to their sk because of the special property of eir A site. The latter is of poor acceptor tivity in the donor molecule itself but n be markedly activated by modification the structure of the substituent on C-1 the A-bearing glycose unit. This points the possibility that suitable analogs of nor systems may exist from which a ınsfer of glycose unit to acceptors could cur but without resulting activation of e A site. In the biological situation, such ıalogs might be expected to abort chain owth. They could prove to be a potent eans of modifying cellular function and ɔrphology.

OPEN DISCUSSION

FRENCH[2]: I should like to discuss just ıe aspect of this very interesting pres-ıtation, namely, the relation between iming by various oligosaccharides and their chain lengths. In the enzyme system of levansucrase, for example, it is apparent that there must be binding sites ("cups") that are specific for the various types of groups found in both the donor and the acceptor (see fig. 1). After transfer of a fructosyl unit to the end of the levan chain, by a simple shift of this chain on the enzyme surface, the fructose unit (which was previously part of the donor molecule) now becomes a part of the acceptor molecule. In this way the acceptor chain really never becomes thoroughly dissociated from the enzyme surface but is allowed to grow as additional sucrose units are used.

If we start out now, however, with a supposed primer (such as F—F—G) for this system this has properties not only analogous to a primer but also analogous to a donor. Then is it not quite reasonable that this potential primer might fool the enzyme into thinking it is really supposed to be a donor instead, and this molecule, instead of fitting into primer binding sites,

[2] Dexter French, Iowa State College.

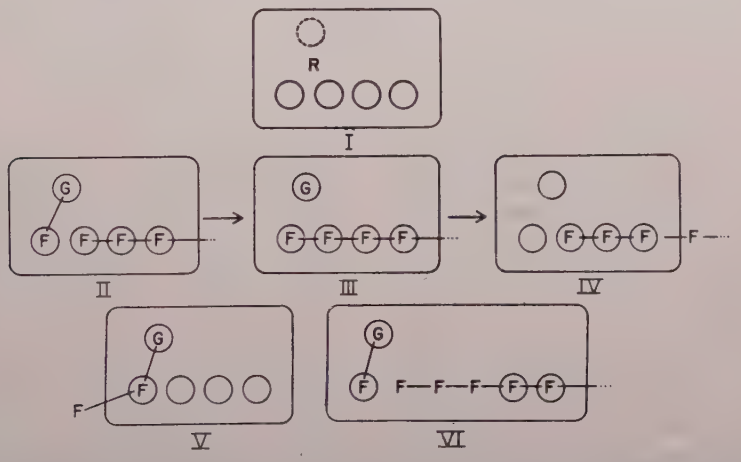

Fig. 1 I. Diagram of levansucrase. The closed circles represent binding sites for fructose units; the dotted circle for a glucosyl (donor) unit; R represents the locus of enzymic groups that effect the transfer.

II. Normal enzyme—substrate primer complex prior to reaction: F, fructose unit; G, glucose unit; F—F—F— is the end of a levan chain.

III. Enzyme—product complex.

IV. Enzyme—primer complex after shift of levan chain by one unit to the right.

V. Enzyme—trisaccharide complex (inhibited) in which the trisaccharide is blocking the donor (sucrose) site.

VI. Enzyme complex showing possible requirement for long-chain primer.

actually fits into donor sites and therefore blocks the synthetic action of the enzyme (see fig. 1)?

HESTRIN: The notion that the enzyme surface has "cups" as visualized in figure 1 is very attractive to us. It could help explain the following specificity feature. In our study of aldoses and their ability to serve at C-1 as acceptor of levansucrase-transferred fructose, we found (as have R. Dedonder and C. Peaud-Lenoel) that a transdisposed hydroxyl pair at C-2 and C-3 is a necessary feature for the reaction. Reduction at C-2 abolished reactivity. Substitution of hydrogen in hydroxyl at C-3 by methyl also abolished reaction. It seems necessary to consider therefore that hydroxyl groups on *both* sides of the sugar ring play a part in determining the specificity of the enzyme.

As to the particular hypothesis presented by Dr. French in relation to the failure of F—F—G to act as an efficient primer, actually levan synthesis from sucrose was not markedly depressed by F—F—G compounds (e.g., 1^F- and 6^F-fructosylsucrose). The picture you proposed, however, suggests to me a possible way a substance such as 6^F-fructosylsucrose could be an intermediate in levan production but at the same time be a poor primer. In the picture the intermediates are in combination with the enzyme and are presumably properly oriented for reaction at their A site. Probably it can also happen that an intermediate is displaced from the protein surface. Such a displacement might be difficult to reverse. Hence the same substances added exogenously might show little or no primer activity. Perhaps an explanation along these lines is applicable as well to the difficulty of reve ing the reaction of levan synthesis. Al although levansucrase can transfer a fr tose unit to alternate hydroxyl sites in acceptor, a reversible shift of the fructo unit from one hydroxyl site to another w not catalyzed detectably by this enzym For example, the isomers 2-, 3-, and 6-fr tofuranosylglucose were each formed transfer of fructose from sucrose to appropriate site in glucose but did not g rise to one another when separately in bated with the enzyme.

It would seem that there may be thin happening that cannot go backward such systems. We should very much l advice on this. Are there thermodynam reasons that would render such an int pretation untenable?

KOSHLAND[3]: I have been fascinated Dr. Hestrin's fine work, and perhaps might comment on two of the many poi that have been brought up.

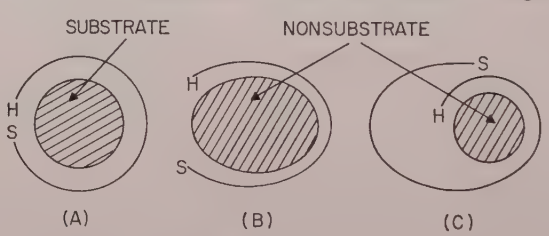

Figure 2

The first involves his evidence that b sides of the pyranose or furanose ring involved in the specificity of carbohydr action. When we first became suspici that the active site might be flexible, considered what we called a "jelly m mechanism. The idea was that the s strate is surrounded by a loop of the zyme molecule in such a way that the quired catalytic groups, H and S, are perfect alignment as in figure 2A. compound that is either too large (as B) or too small (as in C) replaces substrate, this perfect alignment does occur and hence there is no catalysis. mechanism clearly involves both sides

[3] D. E. Koshland, Jr., Brookhaven Nati Laboratory.

ranose ring and is therefore in contrast models in which the substrate "sits n" on the enzyme surface. Although believe the induced-fit hypothesis is a e accurate picture of the steric relations n the "jelly roll" mechanism, the steric tions have many similarities and it is, efore, gratifying to hear evidence that n sides of the carbohydrate rings are mately involved with enzyme.

he second point involves Dr. Hestrin's Dr. French's remarks on "fooling the yme." Dr. J. A. Thoma has been doing ne experiments with β-amylase that sup- t such an idea and finds that Schar- ger dextrins inhibit this enzyme. Our t reaction was surprise since we thought ee terminal 4—OH position would be haracteristic of inhibitors of β-amylase. wever, if a free 4—OH is required for d cleavage but not for binding, it med logical that the enzyme might be acted to interior positions in a long bohydrate chain. Thus, the substrate ld be its own competitive inhibitor. s would normally be obscured in the arent Michaelis constant but can be ed by varying the chain length of the strate. Dr. Thoma's experiments agree h this and therefore support the notion t substrates act as their own inhibitors presenting binding sites that cannot be ved.

HESTRIN: The hexose unit at the chain minals in levan is indeed β-fructo- anosyl. The question is why this ter- al unit cannot be cleaved off hydrolyti- y at a measurable rate by ordinary yeast ertase.

Pursuing a hypothesis similar to that cussed by Dr. Koshland, we thought per- s invertase was trapped with the in- al part of the levan molecule. We nd, however, that levan in solution had appreciable effect on sucrose hydrolysis invertase, nor was invertase removed n the solution when levan was ultra- trifuged from the water phase.

PIGMAN[4]: I have not been too impressed n Dr. French's idea that a chain of donor molecules shifts one unit down the line each time a unit is added. This has been called the "zipper" hypothesis or in this instance the "unzippering" hypothesis. Dr. Koshland's ideas may have merit here and are a part of what we have been thinking. The important factor may be the variable affinity of the active site with the number of units in the chain. The driving force may be the affinity for the enzyme; short units may show an increase in binding ability as the chain increases to an optimal size. Thereafter, the binding might remain fairly constant.

As Dr. Koshland pointed out, long units might be taken up in the middle of the chain and then would block the active site. Such a process might answer the question of why the chains terminate. Why do polysaccharides (and other natural polymers) reach a fairly definite chain length and then stop? By these hypotheses, we might say that the chain growth stops because the probability of a terminus of a chain hitting the active site would become very low as the chain length increased.

CORI[5]: I think you ought to call this mechanism a "musical chair."

HESTRIN: This hypothesis could help to explain how short intermediates may fail to accumulate in a polymerization process, such as one catalyzed by phosphorylase. We would like to know whether we have analogies for such "musical chair" movements of molecules on proteins. If there are such analogies, perhaps we might learn —from the way in which they were discovered—how to demonstrate that such shift of substrate molecules can also occur during polymerization on an enzyme surface.

CORI: I think that in a way Dr. French's idea supports such a movement.

FRENCH: First, one point about the energetics of binding—the specificity of the binding of the substrate to the enzyme is rather high, and the binding energy far exceeds the nonspecific interaction energy

[4] Ward Pigman, University of Alabama.
[5] C. F. Cori, Washington University, St. Louis.

we would expect between such materials as carbohydrate (which is very heavily solvated in water) and protein. So these "cups" are very specific in their attraction for the substrate.

Second, is it possible that the binding site for the acceptor molecule is really quite a long way from the binding site for the donor molecule and, therefore, the primer chain has to be rather long so that the donor can reach it (see fig. 1)?

Another point is this: Some years ago we experimented with *Bacillus subtilis* levansucrase and we did not find all these oligosaccharides you did with your system but rather only the oligosaccharides of the conventional 1,6 type. So it would seem that "levansucrase" is only a generic term and that levansucrases from different microorganisms may have rather widely different specificities.

HESTRIN: In the *Aerobacter levanicum* system, 1^F-fructosylsucrose represents about 95% of all the oligosaccharides formed from sucrose. The other oligosaccharide products (and we did get quite a number) are formed in only very small amounts. Therefore, I would suspect that in *B. subtilis* a reexamination of the situation might likewise reveal similar oligosaccharides. Dedonder and his colleagues showed numerous oligosaccharides formed by the *B. subtilis* enzyme. It remains still to be shown critically whether the major oligosaccharide in this system, as in *A. levanicum* and in a *Corynebacterium* species we have studied, is not 1^F- rather than 6^F-fructosylsucrose.

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actions Involving the Carbon—Nitrogen Bond: terocyclic Compounds

JOHN MACHLIN BUCHANAN, STANDISH CHARD HARTMAN, ROBERT LAWRENCE HERRMANN, AND RICHARD ALLEN DAY

Division of Biochemistry, Department of Biology, Massachusetts Institute of Technology, Cambridge

n the basis of the relatively few en- ic studies pertaining to the synthesis itrogenous compounds, it may still be nature to attempt any generalization he types of reactions responsible for nation of C—N bonds. Nevertheless, iled enzymic investigations of the syn- is of arginine (or urea) and of the nes and pyrimidines have revealed that ain types of reactions do recur, if in ntly varied form, and can be placed in nite categories. Examples may also be wn from the synthesis of other com- nds. The synthesis of arginine is shown eactions (1)-(4) (fig. 1); the syntheses

$$\cdot\ CO_2 + ATP \rightleftharpoons CARBAMYL\text{-}P \quad (1)$$

$$MYL\text{-}P + ORNITHINE \rightleftharpoons CITRULLINE + P_i \quad (2)$$

$$LINE + ASPARTATE + ATP \rightleftharpoons$$
$$INOSUCCINATE + AMP + PP \quad (3)$$

$$INOSUCCINATE \rightleftharpoons ARGININE + FUMARATE \quad (4)$$

Figure 1

urines (fig. 2, 3) and pyrimidines (fig. re shown schematically. Many of the hetic steps in these processes involve nation of C—N bonds between amino pounds and carboxyl groups or car- yl derivatives such as amides or ami- es. These reactions include transfor- ation, transfer of nitrogen from gluta- e and aspartate, ring closures, and ral other processes of amide, amidine, guanidine formation. Many of these tions take place simultaneously with rolysis of a phosphoanhydride bond of ucleoside triphosphate.

Before discussing the similarities among certain reactions in these synthetic processes, it might be well to point out particular instances in which diverse routes have been used to achieve the formation of similar structures. One such example is the syntheses of the ureido groups in citrulline, pyrimidines, and purines. In citrulline and the pyrimidines, the carbon atom and one nitrogen atom of this group originate in CO_2 and ammonium ions via the intermediate formation of carbamyl phosphate, which reacts with the appropriate amino groups of ornithine or aspartate to yield citrulline or carbamyl aspartate. In the purines, the single carbon atom units that eventually become positions 2 and 8 of the ring are introduced at the formate level of oxidation. When enzyme preparations are freed from cyclohydrolase, the enzyme that catalyzes the reaction shown in figure 5, specific formyl derivatives of tetrahydrofolic acid transfer their formyl moieties to the amino groups of glycinamide ribonucleotide and 5-amino-4-imidazolecarboxamide ribonucleotide as shown in figure 2. The formamidine groupings in inosinate and hypoxanthine that result from these transformylation reactions may subsequently be oxidized to the carbamidine (or ureido) level by the appropriate enzymes.

The steps in which cyclization of the pyrimidine ring and the two rings of the purines take place also offer interesting contrasts. Conversion of carbamyl aspartate to orotic acid occurs by linkage of a carbamido nitrogen atom to a carboxyl group without the participation of nucleoside triphosphate. In the final step in the synthesis of inosinic acid from 5-formamido-4-imidazolecarboxamide ribonucleotide

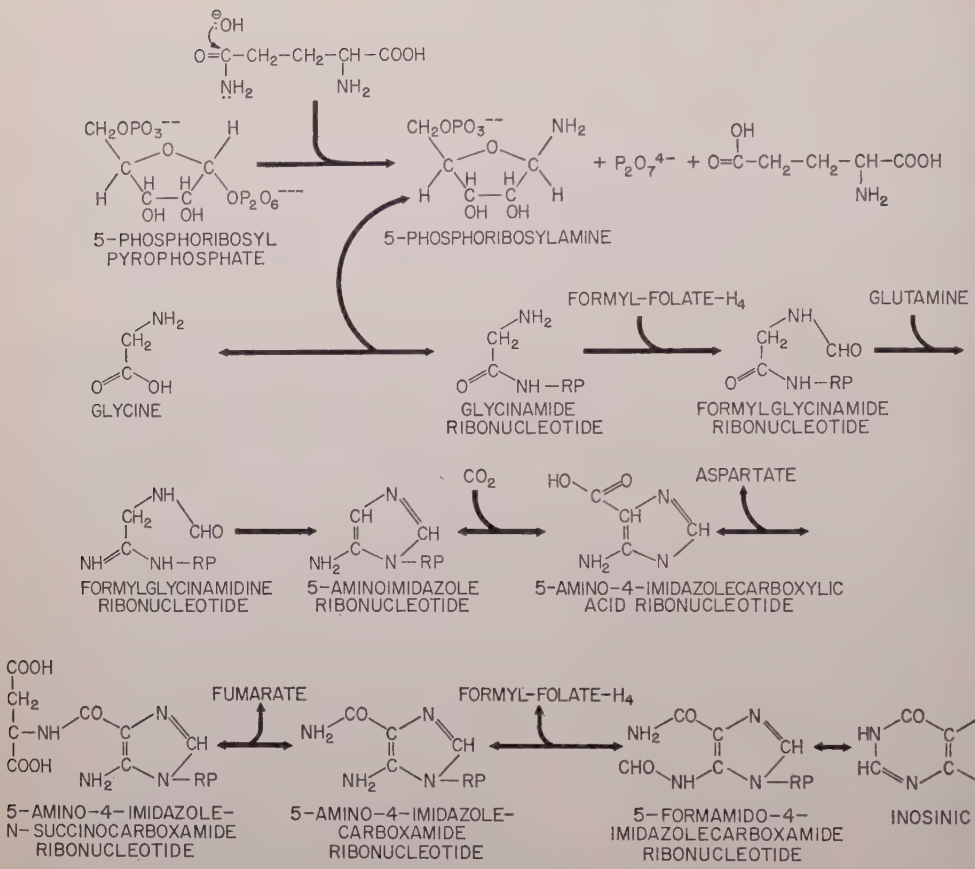

Fig. 2 Enzymic synthesis of inosinic acid.

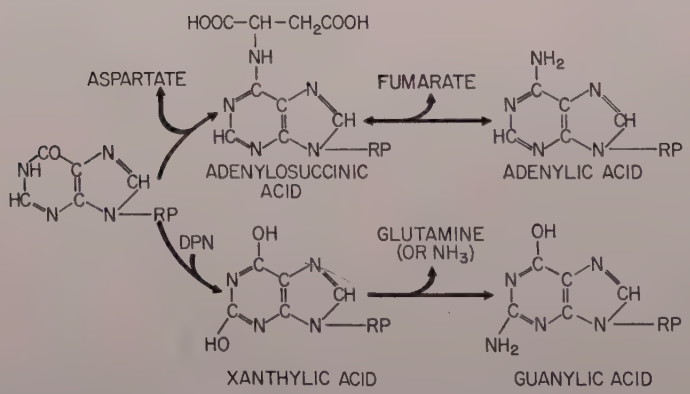

Fig. 3 Enzymic synthesis of adenylate and guanylate from inosinate.

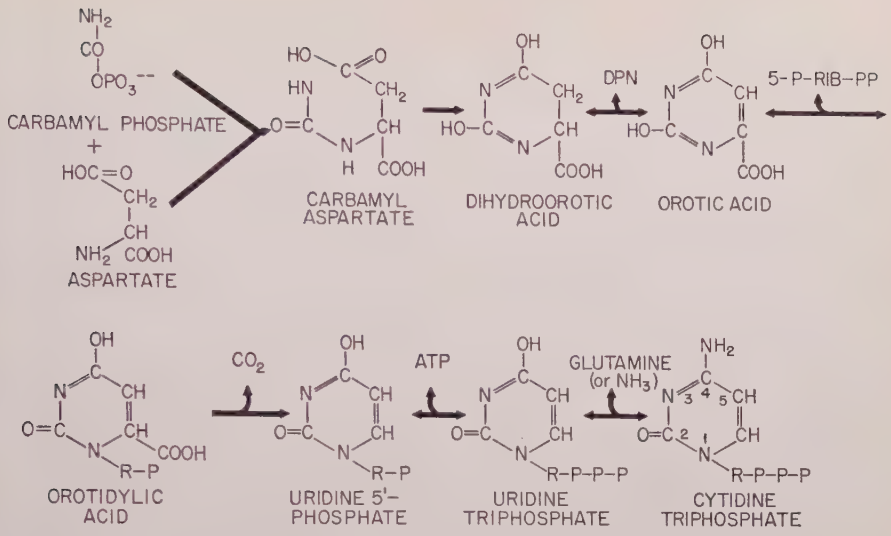

Fig. 4 Enzymic synthesis of pyrimidines.

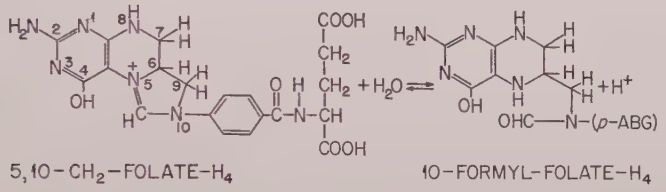

Fig. 5 Action of cyclohydrolase.

(fig. 2) cyclization takes place between amide nitrogen atom and the carbon n of a formamido group. In the analo- s case of the formation of 5-amino- dazole ribonucleotide from formylgly- midine ribonucleotide (fig. 2), how- , the participation of ATP is required. products of ATP utilization are ADP orthophosphate. It is of considerable rest that this one of the three ring- ing reactions discussed should require participation of an energy-yielding pound.

A THEORY OF ENZYME ATALYSIS APPLIED TO FORMATION OF C—N BONDS

lthough variations in the "gross anat- " of the synthetic processes shown in res 1–4 are important, it will be our is here that striking similarities are ountered when many of the reactions —N bond formation are viewed at the l of enzymic mechanisms. Further y of a number of reactions of the three biosynthetic processes indicates that formation of C—N bonds and cleavage of phosphoanhydride bonds may be catalyzed by a single enzyme as an integrated process. These reactions are in contrast to others in which the synthesis of the C—N bond occurs only after an activated intermediate, either free or enzyme bound, has been formed. A new way of depicting the former reactions will be developed in a form that is an extension of the current conception of enzymes as polyfunctional catalysts (Koshland, '54, '56).

These reactions are all of the nucleophilic displacement or substitution type. In the schematic reaction for the synthesis of an amide bond shown in figure 6, a nucleophilic group with an unshared pair of electrons, an amino group, replaces a group at a displacement center. Removal of the group (OH^-) with its unshared electron pair may be facilitated by suitable electron-attracting or electrophilic functions (A^+).

Catalytic action of the nucleophilic and electrophilic groups may conceivably be

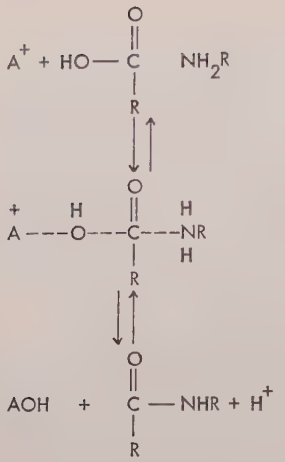

Fig. 6 Schematic representation for the enzymic synthesis of an amide bond.

exerted in two ways: simultaneously, in a concerted manner, or consecutively, in which case an intermediate compound is formed. It is known from examples in organic chemistry that the pathway of a given reaction may depend importantly on the environment. Thus the mutarotation of tetramethylglucose, when catalyzed by the bifunctional catalyst 2-hydroxypyridine in benzene solution, takes place by a concerted mechanism (Swain and Brown, '52a, b). The stepwise process involving intermediate ion formation that may occur in polar solvents is not favored in benzene solution because of the inability of the nonpolar solvent to assist in the formation of such ions. Although the number of instances in which reactions are known definitely to occur by concerted processes are limited in organic chemistry (Bell and Jones, '53), the unique environment of a protein molecule at the point of enzymic action may favor a mechanism of this type.

Whereas enzymic reactions may be c certed, in the strict sense of the term, vestigation of the secondary processes proton transfer are not generally amen to investigation in the enzymic react under discussion. Experimental evide bearing only on the primary bond-form and bond-breaking processes can be tained directly. We will use the term "c certed" to describe reactions in wh these primary events seem to occur sim taneously.

FORMATION OF GLYCINAMIDE RIBONUCLEOTIDE AND RELATED REACTIONS

This discussion centers on the synth of glycinamide ribonucleotide, but re ence will also be made to two other se ingly closely related reactions, those glutamine synthesis from glutamate ammonia and of glutathione forma from γ-glutamylcysteine and glycine 7). These reactions have a great dea common, which warrants discussion them as a group.

$$\text{GLYCINE} + \text{5-PHOSPHORIBOSYLAMINE} + \text{ATP} \rightleftharpoons \text{GLYCINAMIDE RIBONUCLEOTIDE} + \text{ADP} + P_i \qquad (5)$$

$$\text{GLUTAMATE} + NH_3 + \text{ATP} \rightleftharpoons \text{GLUTAMINE} + \text{ADP} + P_i \qquad (6)$$

$$\gamma\text{-GLUTAMYLCYSTEINE} + \text{GLYCINE} + \text{ATP} \rightleftharpoons \text{GLUTATHIONE} + \text{ADP} + P_i \qquad (7)$$

Figure 7

Although the enzyme concerned the synthesis of glycinamide ribonuc tide has been purified 70-fold, there i evidence that more than one enzyme c ponent is required in this reaction or a free intermediate is involved (Gol wait, '56; Hartman and Buchanan, '5 In the presence of magnesium ions, substrates, glycine, ATP, and synth phosphoribosylamine are converted to cinamide ribonucleotide, ADP, and I equivalent amounts. The reverse reac proceeds readily, as evidenced by the mation of equal quantities of glycine ATP from glycinamide ribonucleo ADP, and P_i. An equilibrium constan the reaction could not be obtained bec of the presence of myokinase in the

ne preparation and the apparent de-
uction of the product phosphoribosyl-
ine. Whether, because of the destruc-
n of a product or because of the exist-
; equilibrium of reaction (5), the phos-
orolysis of glycinamide ribonucleotide is
ored over its synthesis.

In the formation of glycine by cleavage
glycinamide ribonucleotide, arsenate is
effective as phosphate as a reactant, but
P is still required (Hartman and Bu-
anan, '58b). Arsenate inhibits incorpor-
on of P^{32} into ATP in the reaction of
cinamide ribonucleotide, ADP, and la-
ed P_i in proportion to the relative
ounts of arsenate and phosphate pres-
, but production of glycine is unim-
red. When the reaction is studied in
forward direction, P^{32}-labeled P_i ex-
anges with ATP only when glycine and
osphoribosylamine are present together.
der the same conditions no exchange
labeled pyrophosphate with ATP occurs.
e enzyme catalyzes formation of a hy-
oxamic acid from glycine, ATP, and hy-
oxylamine. Similarly, a hydroxamic acid
ormed when glycinamide ribonucleotide,
P, and P_i are incubated with NH_2OH.
is reaction is analogous to the amide
oup exchange that the glutamine-synthe-
ing enzyme effects between glutamine,
; Varner and Webster, '55). A similar
ction occurs with glutathione, ADP,
ction occurs when glutathione, ADP,
and NH_2OH in the presence of the
tathione-synthesizing enzyme (Snoke
d Bloch, '55).

The direct coupling between the de-
drative process of amide bond formation
d the hydrolysis of ATP was shown by
O^{18}-transfer reaction (Hartman and
chanan, '58b). Since it was more con-
ient to study the reaction in the direc-
n of glycinamide ribonucleotide phos-
orolysis, O^{18}-labeled P_i was incubated
h glycinamide ribonucleotide and ADP.
e O^{18} content of the glycine formed was
% of that expected for the transfer of
ingle atom of oxygen from P_i to glycine
ring the reaction. In the synthesis of
cinamide ribonucleotide, therefore, it
be concluded that the carboxyl oxygen
m of glycine is extracted by the terminal
osphorus atom of ATP and that, in the
ulting displacement, the terminal phos-
phoanhydride bond of ATP is cleaved. Studies of oxygen transfer in the glutamine-synthesizing system have produced completely analogous results (Levintow *et al.*, '55; Varner and Webster, '55).

Results of studies of glycinamide ribonucleotide synthesis and glutamine and glutathione formation and other processes to be discussed can be summarized as follows: Regardless of the direction in which the reaction is studied, all three reactants are required for any observable reaction. No partial reactions of covalent bond formation or cleavage seem to occur in the absence of the total reaction.

One exception to this is that the enzyme systems that catalyze glycinamide ribonucleotide and glutathione synthesis, but not the glutamine enzyme, carry out an exchange of labeled ADP with ATP in the absence of added substrates. Some consider this exchange reaction to indicate formation of a phosphorylated enzyme that in turn activates the glutamate. Since there is no direct evidence that a phosphorylated enzyme is formed in this reaction and since such a postulated intermediate leads most naturally to mechanisms that contradict the observations concerning these reactions, we have taken the liberty of disregarding this observation in our consideration of mechanism. As in the glutamine-synthesizing enzyme, it may well turn out that further purification of these enzymes may eliminate the ATP-ADP exchange reaction.

We might anticipate that a carboxyl phosphate anhydride, such as glycyl phosphate, would be a likely intermediate in the formation of glycinamide ribonucleotide, particularly in the light of the O^{18} exchange data. It has been found, however, that ADP must be present in the arsenolysis as well as in the phosphorolysis of glycinamide ribonucleotide. On the basis of previous experience with reactions in which arsenate can replace phosphate, we would expect that spontaneous hydrolysis of an arsenate derivative of glycine would obviate the necessity for ADP, if such an intermediate were formed. In the glutamine-synthesizing enzyme, more-direct evidence against the participation of a carboxyl phosphate intermediate has been obtained by Levintow and Meister ('56).

When γ-glutamyl phosphate was added to the enzyme system or was generated from *N*-acetyl-γ-glutamyl phosphate *in situ*, there was no evidence that glutamine was being formed enzymically.

It might be considered that a carboxyl phosphate or carboxyl arsenate intermediate is formed but is protected from hydrolysis by being bound on the enzyme at the site of its formation. This possible scheme would have much in common with the activation of fatty and amino acids, in which cases the acyl adenylates are enzyme-bound intermediates. Such a mechanism does not explain the requirement for nucleoside diphosphate in the reactions in which the amide groups are replaced by NH_2OH, and, in the glutamine reaction, the requirement for ammonia as well as for glutamate in the exchange of ADP with ATP is not accounted for.

As a result of these considerations, Hartman and Buchanan ('58b) proposed a mechanism for the reversible formation of glycinamide ribonucleotide (and by inference, for glutamine and glutathione synthesis, among others) that involves concerted participation of the three reactants at the enzyme site (fig. 8). The carboxyl carbon of glycine undergoes a nucleophilic attack by the nitrogen of 5-phosphoribosylamine at the same time that the terminal phosphorus atom of ATP is exerting electrophilic attraction on one of the oxygen atoms of glycine. In this "push-pu
reaction, the new C—N bond is form
as the C—O bond is broken. ATP clea
to ADP and P_i simultaneously.

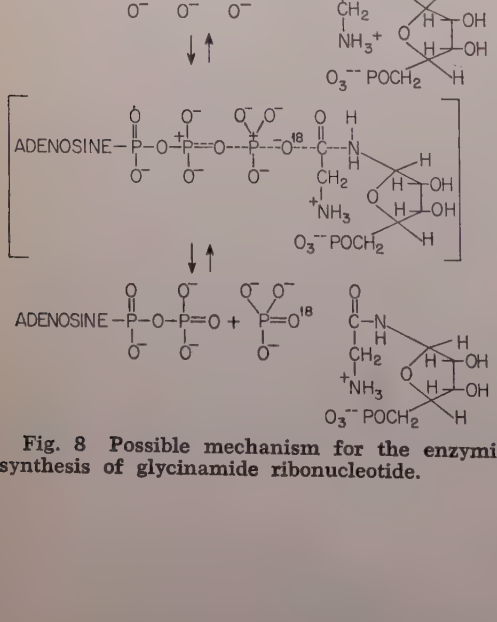

Fig. 8 Possible mechanism for the enzymic synthesis of glycinamide ribonucleotide.

This mechanism is intended to be s
ficiently general and adaptable to se
as an explanation of the bond-formi
processes in a variety of reactions in whi
the particular kinetic patterns may diff
Association and dissociation of the vario
possible enzyme—substrate complexes,
though they may well be rate-limiting ste
in such reactions, are not specified by t
general formulation. We wish to empl
size primarily the concept of concert
participation of the nucleophilic and el
trophilic reactants during these reactio
The name "kinosynthase" was coined
enzymes that catalyze reactions of t
type. It is intended to stress the apparen
direct relation between the synthetic fu
tion of this class of enzymes and the p
ticipation of ATP (or other nucleos
triphosphates) in the reactions. Adopti
of the term kinosynthase (or "kinosynt
tase") for this class of enzymes, howev
should *not* be contingent on the ultim
solution of the mechanism of this
action.

Koshland suggested an alternative fo
for the mechanisms of these reactions t
can account for the experimental findi
and does not require the termolecular
action process, which might be somewh
objectionable on theoretical grounds.
cording to this idea, the presence of
three substrates is required at their
propriate binding positions on the enzy
for the "active site" to be in the pro
configuration for catalysis. This spec
orientation of catalytic groupings on
enzyme will not exist, for example, in
presence of only two substrates. When
three substrates and the enzyme are pr
erly aligned, however, the reaction c
take place in a stepwise (or possibly c
certed) manner. An important conditi
of the stepwise mechanism is that
completion of the covalent bond-formi
steps proceed much faster than the dis
ciation of the products of the partial
actions from the enzyme; otherwise, p
tial exchange reactions would be observ
The decision between these and possi
other mechanisms must be left to the

re and to more-subtle experimental techques.

NITROGEN TRANSFER REACTIONS OF ASPARTATE

There are two examples in the reactions purine biosynthesis and one in those of ginine synthesis in which transfer of e α-amino nitrogen of aspartate takes ace. These transfers occur in two definite eps with the formation of intermediates at contain the carbon chain of aspartate. r example, the nitrogen that becomes nigen atom 1 of the purine ring (Lukens d Buchanan, '57; Miller *et al.*, '57) is corporated by reactions (8) and (9) (fig. see also fig. 2). By a similar series of actions (Abrams and Bentley, '55; Lierman, '56; Carter and Cohen, '56), the mino group at the 6 position of adenylic id is reactions (10) and (11) (see fig.). In reaction (8), an amide bond is rmed between the carboxyl group of the idazole derivative and the amino group aspartate while a molecule of ATP is eaved to ADP and P_i. In all probability is step proceeds similarly to the formaon of glycinamide ribonucleotide from ycine, phosphoribosylamine, and ATP. though the experimental evidence is not complete as in the latter case, the criti- arsenolysis and phosphorolysis experients on the cleavage in reaction (9) have en done. In this case also, there is no action in either direction unless all three bstrates are present. Arsenate will reace phosphate but, as Richard Miller has own, ADP is still required. These relts suggest that the enzyme that catazes reaction (8) will fall into the class the kinosynthases.

The enzyme responsible for the formation of adenylosuccinate undoubtedly belongs in the same category. This reaction is very similar in form to the foregoing, differing principally in that the nucleoside triphosphate is GTP rather than ATP. Lieberman's experiments ('56) showed that there is a transfer of O^{18} from the hydroxyl group at carbon atom 6 of inosinate to P_i in turn was derived from the terminal phosphate of GTP. The question has been raised whether 6-phosphoinosinate might be an intermediate in the conversion of inosinate to adenylosuccinate (Lieberman, '56; Fromm, '58). In the two-step process that formation of this intermediate would involve, only phosphate should be needed for the exchange of labeled aspartate with adenylosuccinate, and GDP might actually be inhibitory by reacting with and removing the intermediate as it is formed. Actually, the presence of GDP stimulated this exchange by over 30-fold. This indicates that participation of all three reactants in the bond-forming steps is necessary and, therefore, a concerted mechanism may be applicable in this reaction.

The second step in these nitrogen-transfer processes [reactions (9) and (11), fig. 9] probably involves a prototropic rearrangement. According to this hypothesis elimination of a proton- and nitrogen-containing group occurs to yield fumarate and 5-amino-4-imidazolecarboxamide ribonucleotide (or adenylate). The analogy between these reactions and those catalyzed by aspartase and fumarase is apparent.

The experimental work of Miller *et al.* ('57) provided evidence that the enzymes

5-AMINOIMIDAZOLECARBOXYLIC ACID RIBONUCLEOTIDE + ASPARTATE + ATP $\rightleftharpoons$
5-AMINO-4-IMIDAZOLE-N-SUCCINOCARBOXAMIDE RIBONUCLEOTIDE + ADP + P_i (8)

5-AMINO-4-IMIDAZOLE-N-SUCCINOCARBOXAMIDE RIBONUCLEOTIDE $\rightleftharpoons$
5-AMINO-4-IMIDAZOLECARBOXAMIDE RIBONUCLEOTIDE + FUMARATE (9)

INOSINATE + ASPARTATE + GTP $\rightleftharpoons$ ADENYLOSUCCINATE + GDP + P_i (10)

ADENYLOSUCCINATE $\rightleftharpoons$ ADENYLATE + FUMARATE (11)

Figure 9

catalyzing reaction (11), adenylosuccinase, and reaction (9), the succinocarboxamide ribonucleotide-cleaving enzyme, may be identical. The same ratio of activities was maintained toward the two substrates during purification of adenylosuccinase from yeast, and each substrate inhibits the cleavage of the other. Furthermore, purine-requiring mutants of *Neurospora crassa, Salmonella typhimurium,* and *Escherichia coli* that lack adenylosuccinase also are unable to split the succinocarboxamide ribonucleotide (Miller *et al.*, '57; Gots and Gollub, '57).

A third reaction of aspartate of this same type, synthesis of arginine from citrulline (Ratner, '54; Ratner and Petrack, '56), takes place according to reactions (3) and (4) (fig. 1). Two minor differences exist between this pair of reactions and the preceding examples. In reaction (3), the products of nucleoside triphosphate utilization are the nucleoside monophosphate and inorganic pyrophosphate. Argininosuccinase is definitely a different enzyme from adenylosuccinase. Despite these differences, this nitrogen-transfer process appears to be essentially the same as the two involved in purine nucleotide synthesis.

TRANSFER OF THE AMIDE NITROGEN OF GLUTAMINE

Aside from the three reactions of purine nucleotide synthesis that will be discussed in some detail, there are at least three other reactions in which glutamine plays a central role as a donor of its amide nitrogen: synthesis of glucosamine from fructose 6-phosphate (Leloir and Cardini, '53; Blumenthal *et al.*, '55), formation of DPN from desamido DPN (Preiss and Handler, '58a, b), and synthesis of cytidylate from uridylate (Kammen and Hurlbert, '58) in mammalian enzyme systems. The three reactions of purine nucleotide synthesis, although containing many similarities, contrast in several important respects.

The first reaction of the latter group (Goldthwait, '56; Hartman and Buchanan, '58a) involves the displacement of the PP group of 5-phosphoribosylpyrophosphate by the amide nitrogen of glutamine according to the top line of figure 2. This reaction, which is catalyzed by 5-phosphoribosylpyrophosphate amidotransferase, is ess tially irreversible. Neither the exchange PP^{32} with 5-phosphoribosylpyrophosph nor the exchange of C^{14}-glutamate w glutamine could be observed.

This reaction probably produces a rib compound of the β configuration from phosphoribosylpyrophosphate, which known to be of the α configuration. Phosphoribosylamine is believed to be the β configuration since it reacts w the other precursors to yield purine cleotides known to be of the β form. D ing the further reactions of 5-phosphori sylamine there is no apparent opportun for inversion.

Several possibilities for the mechani of the reaction (line 1 of fig. 2) have be considered. If we assume that a 5-ph phoribosyl–enzyme complex is involv formation of 5-phosphoribosylamine wou require a double displacement and, in probability, formation of a product of α configuration. If this mechanism w correct then an exchange between PP a 5-phosphoribosylpyrophosphate should cur. This does not occur at a measura rate. Alternatively, a two-step reacti might be envisioned in which an interm diate glutaminyl ribonucleotide is f formed and then hydrolyzed to yield ph phoribosylamine and glutamate. There no conclusive evidence to rule out participation of such an intermediate, presumptive evidence could be ci against its existence. A feasible hypot sis for the mechanism of this reaction that a simultaneous splitting of the am bond of glutamine occurs while the ri sylamine bond is being formed.

By viewing this reaction as a concer process, we may sidestep one theoreti difficulty; namely, that the chemica inert amide nitrogen atom of glutam serves as the primary nucleophilic age Compared to amino nitrogen atoms, th in amide linkage are relatively much l reactive in nucleophilic displacements cause of the effect of the electron-wi drawing carbonyl group to which they attached and the existence of the im tautomeric form of the amide. Howev the nucleophilic reactivity of the amide trogen atom might be enhanced by a sim

aneous cleavage of the amide bond by a econdary nucleophilic displacement at the arboxamide carbon. If this secondary dislacement is caused by a water molecule r a hydroxyl ion, glutamate would be ormed directly. Alternatively, a basic roup on the enzyme could perform this isplacement. In the light of the presumed ction of such groups as the imidazole ring f histidine, the —SH moiety of cysteine, nd the hydroxy group of serine, as funcional groups of certain enzymes (Westeimer, '57; Koshland and Erwin, '57; Schaffer *et al.*, '57; Barnard and Stein, '58; Gladner and Laki, '58; Koshland and Ray, 58), it is tempting to postulate that such group in the polypeptide chain of the nzyme is the nucleophilic agent in these lutamine reactions. The γ-glutamyl derivtive thus formed could then hydrolyze pontaneously. According to this postuate (see fig. 2, line 1), part of the funcion of this enzyme would be that of an midase with an action perhaps similar o that of chymotrypsin or papain. Whereas the acidic function of the enzyme in an midase or esterase reaction might be imply to supply a proton to the "leaving" roup, the corresponding function in the midotransferase system would presumbly be served by the activated carbon tom 1 of phosphoribosylpyrophosphate.

The second reaction of purine nucleotide ynthesis (Levenberg and Buchanan, '57; Melnick and Buchanan, '57) that involves lutamine is reaction (12) (fig. 10; see components representing not more than 30% of the total protein (Herrmann *et al.*, '59). So far as is known, only one enzyme is responsible for the entire reaction. Likewise, no intermediates of the reaction have been indicated. Attempts to demonstrate the reversibility of the reaction have been unsuccessful. Neither P^{32}-labeled P_i nor C^{14}-glutamate exchanges with ATP or glutamine, respectively.

It seems likely that the mechanism of this nitrogen transfer (fig. 11) is similar to that occurring in the phosphoribosylpyrophosphate amidotransferase reaction. One apparent difference is the direct participation of ATP in the formation of formylglycinamidine ribonucleotide; whereas, in the synthesis of phosphoribosylamine, a phosphorylation step occurs separately from the amido transfer. It is proposed that the electrophilic agent, ATP, exerts its effect in the present case not through a direct covalent linkage, as in phosphoribosylpyrophosphate, but rather by a partial electronic interaction resulting from the proximity (on the enzyme site) of the ATP with the group to be displaced. Presumably, activation of the nucleophilic reactant, glutamine, would result from a displacement by a basic group, much as in the proposed mechanism for the synthesis of phosphoribosylamine.

Because of the irreversibility of the glutamine reactions, it is not possible to apply the criterion of the arsenolysis experiments to determine whether a concerted

$$\text{FORMYLGLYCINAMIDE RIBONUCLEOTIDE} + \text{GLUTAMINE} + \text{ATP} + H_2O \longrightarrow$$
$$\text{FORMYLGLYCINAMIDINE RIBONUCLEOTIDE} + \text{GLUTAMATE} + \text{ADP} + P_i \qquad (12)$$

$$\text{XANTHYLIC ACID} + \text{GLUTAMINE} + \text{ATP} + H_2O \longrightarrow$$
$$\text{GUANYLIC ACID} + \text{GLUTAMATE} + \text{AMP} + \text{PP} \qquad (13)$$

Figure 10

lso fig. 2), which is essentially irreversble, as are all other reactions of glutamine o far studied. This enzyme, which might e called formylglycinamide ribonucleotide midotransferase, has been purified now bout 2000-fold from chicken liver and is ure except for two small contaminating reaction occurs, as has been done in two previous instances. However, since the reaction occurs in the forward direction in the presence of a single highly purified enzyme without the appearance of free intermediates, it may be that this reaction, which coordinates the utilization of energy

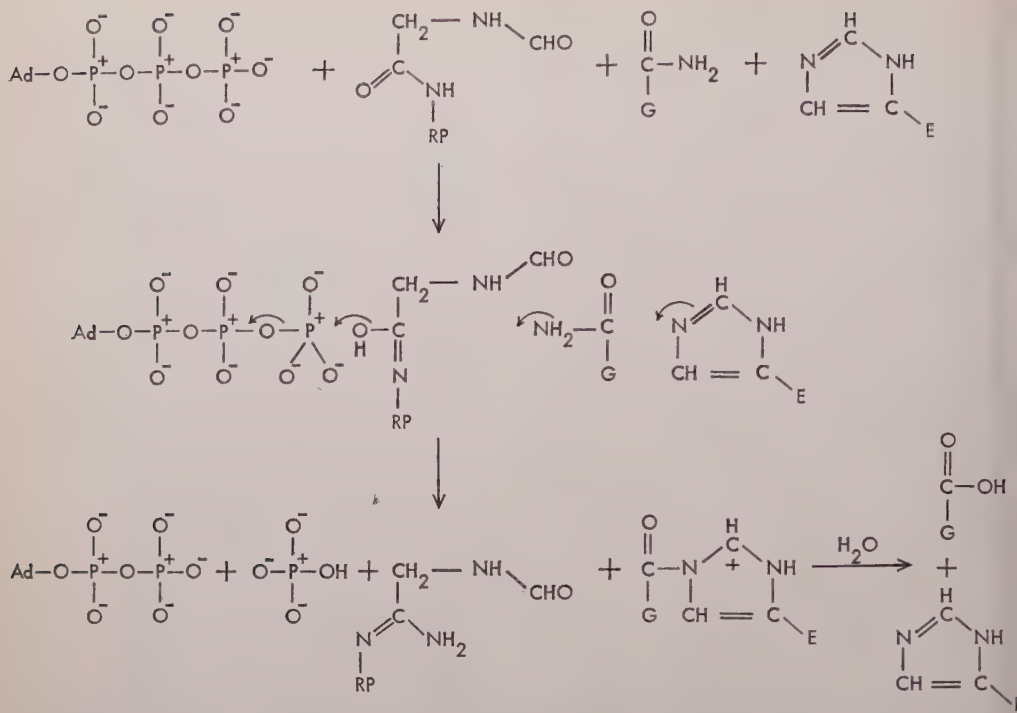

Fig. 11 Possible mechanism for enzymic synthesis of formylglycinamide ribonucleotide. G, glutamyl group; E, imidazole group, a hypothetical reactive hystidyl residue of the enzyme.

from the hydrolysis of a phosphoanhydride bond with the synthesis of a C—N bond, is of the same type as those reactions discussed previously. The problem of obtaining experimental evidence in support of or in opposition to these proposed mechanisms is a difficult one. They are almost entirely hypothetical, representing what seems to be the simplest interpretation of the experimental findings in terms of a plausible theory of enzyme catalysis.

The third glutamine reaction of purine nucleotide synthesis is the conversion of xanthylic to guanylic acid (Abrams and Bentley, '55, '59; Moyed and Magasanik, '57; Magasanik *et al.*, '57; Lagerkvist, '58a, b) (see fig. 3). The enzyme from pigeon liver that catalyzes the reaction between xanthylic acid, glutamine, and ATP has been purified about 90-fold. Lagerkvist ('58a, b) has studied the mechanism of this conversion. As shown in reaction (13), the products of ATP utilization are AMP and PP. Magnesium ions are required. When the purified enzyme is used, the requirement for glutamine cannot replaced by L-asparagine or L-glutama Ammonia is reactive in this system, b the concentration required for saturati is 200 times that required for glutamin and maximum activity is only 15% great.

Although the enzyme preparation w not catalyze exchange of glutamate wi glutamine, PP exchanges with ATP. Th latter exchange reaction was not deper ent on further additions of substrate oth than the PP and ATP. Since the enzym systems responsible for reaction (13) a the PP exchange are partially separable a chromatographic procedure, there is go reason to believe that this exchange rea tion is not an activity of the guanylic ac synthesizing enzyme (xanthylic acid an dotransferase).

The transfer of O^{18} from reactants products has also been reported. Wh xanthylic acid was labeled at the 2 positi with O^{18} and incubated with ATP a glutamine, O^{18} transferred stoichiometr

lly to the phosphate of AMP. This sug-
ested that the intermediate formation of
n adenyl-xanthylic acid may occur in this
eaction in analogy to the adenyl amino
cids formed in amino acid activation. In
uch a mechanism, PP should exchange
ith ATP as an integral activity of the
minating enzyme, and this exchange
hould depend on the presence of xanthylic
cid. Since this is not the case, there is no
ood evidence for the existence of the
denyl-xanthylic acid intermediate.

Abrams and Bentley ('59) have shown
complete analogy between a mam-
nalian system and the avian system stud-
ed by Lagerkvist, except that —SH com-
ounds seem to be required by the calf
hymus enzyme. They conclude that the
ost likely mechanism for this aminating
eaction involves a simultaneous interac-
ion of the substrate xanthylic acid with
he electrophilic agent (ATP) and the nu-
leophilic agent (glutamine). In the en-
ymic system isolated from *Aerobacter*
erogenes and studied by Moyed and Mag-
sanik ('57) and by Magasanik *et al.* ('57),
mmonia was used as the nitrogen donor
ather than glutamine in the synthesis of
uanylic acid.

The mechanism for the glutamine-de-
endent amination of xanthylic acid may
e similar to that shown in figure 11 for
he amination of formylglycinamide ribo-
ucleotide. AMP and PP are produced in
his reaction, however, instead of ADP and
. This and the information from the O^{18}
xperiment show that the nucleophilic dis-
lacement on ATP must occur at the inner-
ost phosphorus atom rather than at the
erminal one. The bacterial amination re-
ction may proceed in a simpler manner
ince the secondary nucleophilic displace-
ent required to break the amide bond in
he glutamine reaction can be dispensed
with. This simplification may be more apparent than real, however, since a proton must be removed from ammonia in the formation of the amino group. It seems likely that this function is performed by a basic group at the active site.

STUDIES WITH ANTIMETABOLITES OF GLUTAMINE

A development of considerable potential importance in the chemistry of amide transfer reactions at the enzyme site is the discovery that the amidotransferases may be inhibited by the antibiotics, azaserine (*O*-diazoacetyl-L-serine) or 6-diazo-5-oxo-L-norleucine (DON) (Levenberg *et al.*, '57) (fig. 12). Both compounds behave as antimetabolites of glutamine. Their action has now been studied in five reactions (Levenberg *et al.*, '57; Preiss and Handler, '58b), and values for the K_m of glutamine and the K_I of the inhibitors have been reported for four of these reactions (table 1). Of these reactions, the conversion of formylglycinamide ribonucleotide to formylglycinamidine ribonucleotide is most importantly affected by azaserine. The inhibition constant of azaserine for this reaction is 3.4×10^{-5} *M*, whereas corresponding values for the other reactions range from 1.3×10^{-3} to 6.7×10^{-3} *M*. Still more important, the ratio of the K_m of glutamine to K_I of azaserine for the formylglycinamide ribonucleotide reaction is 18, a value several times greater than that for any of the other reactions. This means that, for a given concentration of glutamine, azaserine is relatively more potent as an inhibitor for this one reaction than for the others. In the one instance, however, where DON was examined, there was a close correspondence in the ability of this antimetabolite to inhibit both of the glutamine reactions of inosinate synthesis

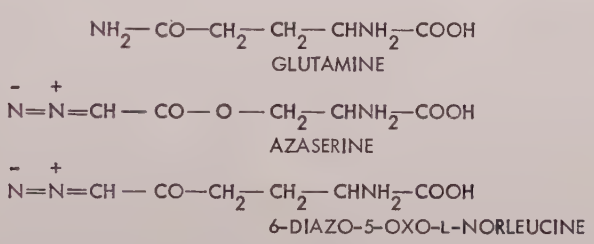

Fig. 12 Antimetabolites of glutamine.

TABLE 1

Constants for the competitive inhibition of glutamine-requiring enzymes by azaserine and 6-diazo-5-oxo-L-norleucine

Reaction	K_m for glutamine	Azaserine		6-Diazo-5-oxo-L-norleucine	
		K_I	K_m/K_I	K_I	K_m/K_I
Desamido DPN → DPN	3.5×10^{-3}	1.3×10^{-3}	2.7	Not determined	
Xanthylic → guanylic acid	4.6×10^{-4}	6.7×10^{-3}	0.07	Not determined	
Phosphoribosylpyrophosphate → phosphoribosylamine	1.3×10^{-3}	4.6×10^{-3}	0.25	2.2×10^{-6}	680
Formylglycinamide ribonucleotide → formylglycinamidine ribonucleotide	7.0×10^{-4}	3.4×10^{-5}	18	1.1×10^{-6}	730

de novo. This may reflect that DON more closely related structurally to glu amine than is azaserine and that the a proach of the latter substance to the si of reaction in some enzymes may be mo difficult sterically than in others.

Another interesting feature of the stru tural relations of these antimetabolites that small changes in the composition the compounds may result in complete partial loss of their inhibitory activi (Buchanan, '58). Since the D forms azaserine or DON are inactive, the stere configuration of the antimetabolites mu be important. Carbamyl glutamine and diazo-4-oxo-L-norvaline are likewise unab to inhibit the enzyme, a demonstratio that both the presence of the function diazo group and the structure of the sid chain are important in the ability of co pounds to inhibit these reactions.

Both of the glutamine enzymes of in sinate biosynthesis have been extensive purified by a combination of steps invol ing ammonium salt and ethanol fraction tion together with purification on hydrox apatite and diethylaminoethyl cellulo columns.

We have tried to determine in prelim ary form some of the constants and cha acteristics of formylglycinamide ribon cleotide amidotransferase. In the ultr centrifuge, it has a sedimentation consta of 10.1×10^{-13}, a value that would indica a molecular weight of about 200,000. the absence of its substrates, the purifi amidotransferase is rapidly inactivated incubation at 38°C. for even a few m utes. If it has been fractionated in t presence of glutamine, however, it is longer sensitive in this manner, at le not to the same degree. Likewise, samp that have stood at 3°C. for 48 hours the absence of P_i completely lose th activity. The amidotransferase is also s sitive to typical —SH reagents such as chloromercuribenzoate at concentratio of $\sim 5 \times 10^{-5}$ *M* and is inhibited by io acetate, ferricyanide, and *N*-ethyl ma mide at concentrations of 10^{-3} to 10^{-4} In addition, NH_2OH at 5×10^{-3} and di propylfluorophosphate at 2×10^{-4} *M* hibit the enzyme activity by 27 and 40 respectively.

BINDING OF FORMYLGLYCINAMIDE RIBONUCLEOTIDE AMIDOTRANSFERASE WITH RADIOACTIVE AZASERINE

Radioactive azaserine with C^{14} in the dicated position ($N_2C^{14}HCOOCH_2CHNH_2$ OOH) was prepared from glycine 2-C^{14} the method of Nicolaides *et al.* ('54). hen incubated with the partially purified nidotransferase, the enzyme binds radio- tive azaserine in proportion to the loss of zyme activity. Binding is measured af- r the enzyme is treated with sodium boro- ydride and dialyzed. Apparently this ocess is necessary to remove extran- usly bound azaserine. In a control ex- riment we found that bovine albumin d not bind azaserine. According to pres- t estimates, ~1 mole of radioactive aza- rine is bound in covalent linkage per ole of enzyme.

This C^{14}-labeled enzyme has now been gested with chymotrypsin, among other oteolytic enzymes, and three radioactive lypeptides have been isolated by paper ectrophoresis. One of the radioactive eas contained 50% of the radioactivity tracted from the paper; the other two ntained 25% each. Digestion of the ^{4}-labeled enzyme with acid gives a radio- tive product that is eluted from a Dowex-) resin column in the fractions just pre- ding those containing the dicarboxylic ids. This compound was purified by fur- er paper chromatography. Migration of is compound in various solvents was mpared with migrations of 5-carboxy- ethylcysteine and glycolic acid. These sults indicate that the unknown com- und may be 5-carboxymethylcysteine, t this finding requires further verifica- n.

Finally, one further characteristic of hibition of the amidotransferases by aza- rine should be mentioned. As Preiss and ndler ('58b) showed, the desamido DPN idotransferase is inhibited by azaserine ly in the presence of the other compon- ts of the reaction, desamido DPN, g^{++}, K^+, and ATP. This relative depend- ce of the inhibition on the presence of e other materials differs from the situ- on with the formylglycinamide ribonu- eotide amidotransferase, which is very nsitive to azaserine even in the absence the other substances. However, the ac-

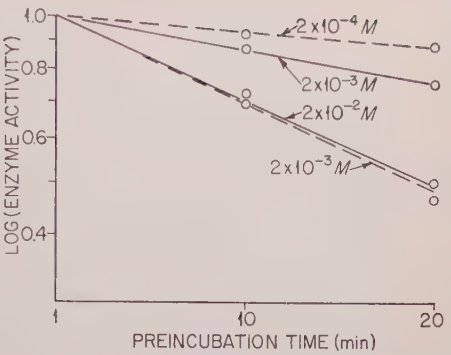

Fig. 13 Rate of reaction of azaserine with 5-phosphoribosylpyrophosphate amidotransferase. Broken lines, with 5-phosphoribosylpyrophosphate; solid lines, without it.

tion of azaserine on the 5-phosphoribosylpyrophosphate amidotransferase has now been found to have a marked dependence on the presence of 5-phosphoribosylpyrophosphate (fig. 13). In the presence of azaserine alone, there is slow and irreversible inactivation of the enzyme, the rate of which may be increased approximately tenfold in the presence of 5-phosphoribosylpyrophosphate.

These data together with those of Preiss and Handler indicate that, for inactivation of the amidotransferases by these antimetabolites, all components of the reaction system (glutamine of course excluded) must be aligned in proper position on the enzyme site before reaction takes place. The inactivation reaction that occurs in the presence of azaserine and the substrates may be analogous to the normal reaction that occurs when glutamine replaces azaserine in the system. These findings might be cited as further evidence that substrates may induce formation of the catalytic configuration of the active site of the enzyme. The very specific nature of the inhibitor-enzyme interaction is also suggested.

OPEN DISCUSSION

NOVELLI[1]: I should like to raise a possible question about introducing the term "kinosynthases" in particular to the reaction in which glycine participates. This is being proposed on the basis of (1) a requirement for ATP in the synthases of the glycinamide ribonucleotide and (2) of the

[1] G. D. Novelli, Oak Ridge National Laboratory.

failure to observe the accumulation of any intermediate. I would like to recall some experiments with the synthesis of acetyl—CoA that might have led to the same formylation were it not that an exchange reaction was possible. The reaction of ATP plus CoA plus acetate could be written as a kinosynthase, since all components are required to form the compound; but it can, in fact, be written as two steps since the first one is the activation of acetate and in this case requires the liberation of pyrophosphate. What made it possible to break this into two steps was the exchange of PP with ATP in the presence of acetate, and in the absence of CoA, to form acetyl—adenylate. This compound is considered to be tightly enzyme bound and there was a good deal of discussion about its existence until a sufficient amount of enzyme could be used to demonstrate the actual formation of the compound.

Dr. M. J. Cormier in our group isolated a glycine-activating enzyme from *Photobacterium fischeri* that might be the same system you are examining. We were using hydroxylamine as the acceptor of the activated glycine, and this could be formulated as yielding an enzyme-bound glycyl phosphate. The O^{18} experiment indicates a transfer of the O^{18} from the carboxyl group of glycine to P_i. My question was is it possible that you actually do have a two-step reaction. If your enzyme were free of myokinase, you would then expect to get ADP and ATP exchange in the absence of acceptor amine.

BUCHANAN: I can best answer your question by using the glutamine synthetase system as an example. In this system glutamic acid, ATP_3, and NH_3 react to yield glutamine, ADP, and P_i. The reaction is reversible. This enzyme differs in several important respects from the acetate-activating system you just mentioned. First, with highly purified glutamine synthetase, there is little or no exchange of ADP with ATP unless both glutamic acid *and* ammonia are present. Likewise P_i^{32} does not exchange with ATP unless glutamine and ADP are present. This then differs from the acetate-activating system where exchange of PP with ATP occurs in the presence of acetate but in the *absence* of CoA.

Again, on the other end of the gl amine synthetase reaction, the exchan of the amide nitrogen of glutamine quires the presence of ADP and P_i as dc the formation of the hydroxamate fr glutamine. A. Meister and colleagues ha shown that free glutamate is not an termediate in the formation of γ-glutam hydroxamate from glutamine in the pr ence of ADP, P_i, and hydroxylamine. these experiments argue against the exi ence of a covalently bonded intermedia in the glutamine synthetase system c responding to adenyl-acetate in the aceta activating system.

Perhaps I should also mention the rece findings of R. Abrams and M. Bentley, w have been studying the following reacti in thymus: xanthylic acid + glutamine ATP + H_2O → guanylic acid + glutam acid + AMP + PP. In crude extracts exchange between PP and ATP cou be observed that suggested possibly th an adenyl—xanthylic complex was intermediate of the foregoing reactic However, as the system became pu fied, the enzymic component respon ble for the PP-ATP exchange could be tirely separated from the xanthylic amic transferase reaction. The two systems th have no relation to each other. Howev we might have been misled concerni possible intermediates of this reaction such a separation had not been achiev It is therefore dangerous to generalize present concept of the mechanism of acetate-activating enzyme to other syste that are undoubtedly different from it.

To return to the initial question rais by Dr. Novelli concerning the usefuln of introducing the term "kinosynthase" "kinosynthetase," I believe there is so advantage of setting aside this group enzymes or enzyme reactions of enigma mechanism in a separate category. these reactions there is the synthesis o new bond, such as a C—N bond, with simultaneous splitting of ATP to ADP a P_i or to AMP and PP. These enzymes not kinases of the ordinary type. Rega less of the ultimate decision as to mechanism of these reactions, they similar and should be identified as a gro

BROWN[2]: We have been studying the iosynthesis of various vitamins and coen-ymes that include a number of enzymic eactions by which C—N bonds are ormed. As Dr. Buchanan mentioned, the ne about which we have the most infor-nation is the formation of the C—N bond y which the pyrimidine moiety of thia-

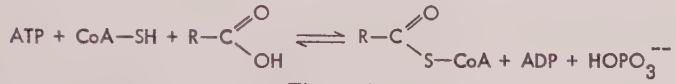

Figure 14

ine is attached to the thiazole portion of ne molecule. The mechanism of the for-nation of this bond seems to be quite dif-erent from any of the mechanisms Dr. uchanan described. I would like briefly o indicate how this bond is formed be-ause the mechanism might be important s a general mechanism in the formation f similar C—N bonds in other compounds.

We used enzymes prepared from cell-ee extract of baker's yeast. The total ctivity for thiamine synthesis has been eparated into two enzyme fractions. An nzyme, or enzymes, present in the first raction catalyzes the conversion of 2-nethyl-4-amino-5-hydroxymethyl pyrimi-ine, ATP, and Mg^{++} to the pyrophos-horic acid ester of the pyrimidine. The econd enzyme fraction catalyzes the con-ersion of this ester and thiazole to a com-ound that behaves like thiamine in micro-iological assays. It has been reported that synthetic phosphomonoester of this py-midine could serve as a substrate for the ynthesis of thiamine in the presence of hiazole. We feel that probably the en-yme system that was used in this case as contaminated with ATP, because the hosphomonoester cannot serve as sub-rate in our system unless ATP and the roper enzyme are supplied to convert the nonoester to the pyrophosphoric ester.

HAGER[3]: I think that a possible test ould be applied to the concerted mech-nism that you have suggested for these eactions where three reactants are in-olved, and that would be where you used oubly labeled phosphate (P^{32},O^{18} phos-hate) and studied the rates of exchange f these two labels into their respective ubstrates. If a concerted mechanism were involved, it would have to be a nonstop process, so that the exchange rates of O^{18} and P^{32} would have to be identical.

I would like to point out that in one case this has been done; namely, in the succinic kinase reaction where we have an analogous case with three reactants and three products according to figure 14. Following Dr. Mildred Cohn's lead where she showed that the O^{18} of P_i would transfer to the carboxyl group of succinate, we incubated P^{32},O^{18}-labeled phosphate and purified succinic thiokinase together with the other reaction components and showed that the exchange rate of O^{18} into succinate is some four to five times as great as the exchange rate of P^{32} into ATP. Although this result does not indicate what the intermediate in this reaction might be, it does predict that an intermediate exists and that this intermediate will allow O^{18} exchange without P^{32} exchange. Consequently, this result would predict that this reaction does not involve a concerted mechanism.

BUCHANAN: I believe the results you obtained with O^{18} and P^{32} exchange with the succinic thiokinase system are not at variance with the concept of a concerted reaction or of the existence of a transition complex on the enzyme.

In a system in which glycine, ATP, and 5-phosphoribosylamine are being formed from glycinamide ribonucleotide, ADP, and P_i, it might be expected that one O^{18} of P_i is incorporated into the carboxyl carbon of glycine for every P^{32} of P_i incorporated into ATP. Essentially, within the limits of error, this is what we find.

However, if your results were obtained in a system poised at equilibrium, a higher exchange of O^{18} of doubly labeled P_i into the carbonyl carbon than of P^{32} into ATP might well be expected. Again I would like to refer to the glutamine synthetase system since much more kinetic information

[2] G. M. Brown, Massachusetts Institute of Technology.

[3] L. P. Hager, Harvard University.

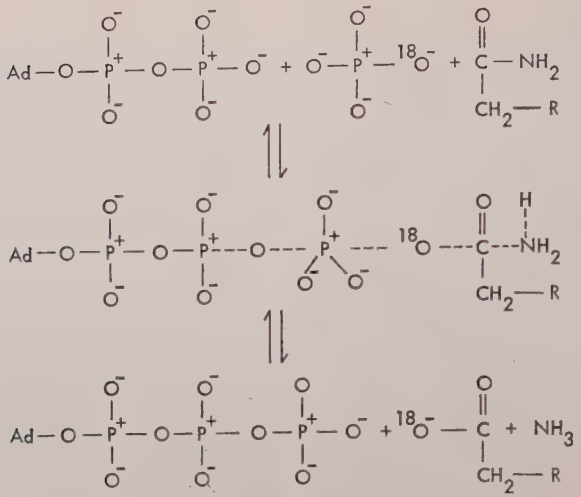

Figure 15

is available from this system than from our own. Let us assume that, in figure 15, the substrates react at the enzyme surface (A) to form a complex (B) that then may break down to products (C). For simplification, we assume that the substrates may occur on the enzyme surface in states A, B, or C. In addition, there are dissociations of products and reactants from the surface of the enzyme and replacement of these substances by their counterparts in solution. Thus, to achieve a greater equilibration of O^{18} of doubly labeled P_i with the γ-carboxyl carbon of glutamic acid as compared with the equilibration of P^{32} of P_i with ATP, we must only assume that the dissociation of ATP from the enzyme surface is considerably slower than the dissociation of glutamic acid and P_i.

Boyer and his colleagues have in fact carried out extensive studies on the exchange of P^{32}-labeled P_i with ATP and of C^{14}-glutamic acid with glutamine in the glutamine synthetase system at equilibrium and have obtained quite variable results. Depending on the relative concentrations of P_i and ATP in respect to the concentrations of glutamic acid and glutamine, the exchange of P_i^{32} may actually be greater than, equal to, or less than the exchange of C^{14}-glutamic acid. Boyer concluded that pathways of reactions cannot be determined by such isotope-exchange studies and that the association and dissociation of substrates from the enzyme surface a the limiting steps and *not* interactions substrates that occur on the enzyme s face. It seems to me, therefore, Dr. Hag that O^{18}- and P^{32}-exchange studies can establish or rule out any concepts of t interactions at the enzyme surface, at lea on the basis of the information now ava able.

HAGER: If the reaction is concerted this point, whether or not these assoc tions occur, the only way that the act exchange can occur is for it to start a to go through. I believe that, if you ins on concerted mechanism, you have to ha identical exchange rates of all the possi reactants with allowances, of course, the dissociation constants of the enzyme substrate complexes vary greatly.

BUCHANAN: I believe we must consi very seriously the possible differences t do exist in the association and dissociat of substrates from the enzyme surface

METZLER[4]: I think that Dr. Koshla will present evidence in his paper to s port the idea that the binding of the s strate affects the configuration of the zyme and actually helps to create the tive site. This might explain the beha of an enzyme such as glutamine synt tase where ATP, glutamic acid, and monia all have to be bound simultaneou

[4] D. E. Metzler, Iowa State College.

o not want to give up the idea of two
ısecutive displacement reactions—the
t between ATP and the carboxyl group
l the second between the bound acyl—
ɔsphate intermediate and ammonia.
haps the binding of the ATP is essen-
l for the activity of the second site be-
ıse of a change in the configuration of
enzyme, and the binding of the am-
nia is essential for the configuration at
first displacement site. Then no reac-
ns will occur until all three substrates
present on the enzyme surface.

I should also like to ask a question re-
ing to Dr. Brown's findings on thiamine
synthesis and to various other reactions
which PP is produced. Dr. Lipmann, in
article in Science, commented on the
ɔduction of PP in relation to the energet-
of sulfate activation. I should like to
k him whether the cleavage of PP to P_i
these sequences that have been pre-
ıted is of metabolic significance.

BUCHANAN: In regard to Dr. Metzler's
st comment, we have seriously consid-
d Dr. Koshland's suggestion that the
aracteristics of the kinosynthase reac-
ns may reflect the role the substrates
y in establishing the proper configura-
n of the enzyme at the enzyme site. As
ntioned in our paper, it is possible that
need for all substrates regardless of the
ection of the reaction may be related to
ir effect on enzyme configuration. If
assume that the only reason for the
ed of all substrates involves their role in
zyme configuration, then it is no longer
cessary to require that the substrates re-
on the enzyme surface in a concerted
ınner but that a stepwise process might
ve equally well. This is certainly a
stifiable interpretation of the data. We
ıst, however, keep in mind that the con-
ɔt of the "configuration of the enzyme
e" is still a hypothesis, albeit an extreme-
interesting one. As one last thought,
wever, it is a plausible suggestion that
process of bringing the enzyme into
ɔper configuration is one step in achiev-
the conditions required to permit the
ıction to take place in a concerted fash-
and that the two concepts, i.e., enzyme
ıfiguration and concerted reactions, are
t to be considered as alternative hypo-
theses but actually as parts of the same hypothesis.

HANDLER[5]: The enzyme responsible for the final step in the biosynthesis of DPN, DPN synthetase, appears to be a hybrid of several of the enzyme types that Dr. Buchanan discussed and so warrants inclusion in the present discussion.

The reaction catalyzed by this enzyme is as follows: nicotinic acid–adenine dinucleotide + ATP + glutamine → DPN + AMP + glutamate + PP_i. Thus, as in the glutamine synthetase reaction, the product is an amide. However, the energy of the ATP is made available by a process that results in the formation of AMP + PP_i rather than ADP + P_i. There are also a number of other interesting properties of this system. The reaction will proceed with either glutamine or ammonia as the nitrogen donor. K_m for NH_3 is about one-half K_m for glutamine. In the mammal, however, this is essentially without meaning. The K_m for glutamine is of the same order as the concentration of glutamine observed in such tissues as liver, heart, and brain. It would be impossible, however, to achieve a concentration of ammonia in animal cells sufficient to permit this reaction. Whereas K_m for NH_3 is about 5×10^{-4} M, to achieve this in mammalian cells would require that the NH_4^+ concentration be about 0.05 M at pH 7.4 whereas its actual concentration is about 10^{-5} M and the NH_3 concentration is no more than 10^{-7} M—no more than one one-thousandth the K_m concentration for the species NH_3. Accordingly, glutamine must be the normal nitrogen donor when this reaction proceeds under physiological circumstances.

An ever-increasing number of reactions have been observed in which the amide nitrogen of glutamine is transferred to another carbon chain. The foregoing remarks suggest the peculiar physiological role of glutamine in this regard. Glutamine seems to be the only means physiologically available to bring up to an enzyme surface a nitrogen at the oxidation level of ammonia but that is not protonated and bears no net charge. The amide nitrogen of glutamine, like that of ammonia, car-

[5] Philip Handler, Duke University.

ries an unshared pair of electrons that are free to engage in the enzymically catalyzed reaction. In this sense, therefore, glutamine apparently serves as "active ammonia" for many biological reactions. There seem to be at least two other known instances in which glutamine and ammonia can serve in equivalent fashion as nitrogen donors, and it is only the extremely low steady-state concentration of ammonia that necessitates participation of glutamine in these reactions.

The enzyme also exhibits one other interesting aspect. Like other glutamine-dependent reactions, DPN biosynthesis, with glutamine as the nitrogen donor, is inhibited by azaserine and by DON. When tested in the usual fashion, the inhibition by azaserine appears to be uncompetitive with respect to glutamine, and indeed, as the reaction proceeds, the extent of inhibition increases steadily. Preincubation of the enzyme with azaserine does not appreciably affect the kinetics of the subsequent process. However, if the enzyme is preincubated with azaserine together with ATP and nicotinic acid–adenine dinucleotide for a few minutes before addition of the glutamine, the enzyme appears

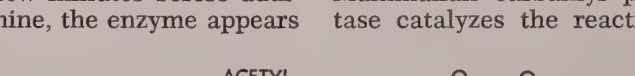

Figure 16

to be completely inhibited. Thus azaserine appears to be extremely tightly, if not irreversibly, bound at the normal binding site for glutamine. However, a necessary condition for such binding is the presence on the active site of the other components of the reaction mixture, ATP plus nicotinic acid-adenine dinucleotide, or possibly a product of their interaction. This observation clearly bears on the proposition raised earlier that it is the presence on the enzyme surface of two of these substrates that determines the surface conformation of the enzyme thereby permitting the binding of the third substrate or its competitive inhibitor. Particularly noteworthy is the fact that azaserine under these conditions is without effect on the rate of DPN synthesis with ammonia as nitrogen donor, presumably suggesting that there is no "binding site" on the enzyme surface ammonia *per se*.

LIPMANN[6]: I think Feodor Lynen actually the first to suggest the role PP in these reactions, which we might occur by PP elimination. Now when PP participates in the backward reacti as, for example, in the formation of (adenosine monophosphosulfate), this very important because a backward re tion in this case is favored and we wo not really get any APS if there were no present. If some similar situation ex in the case Dr. Buchanan mentioned I not really know.

I have been thinking that some might say something about the simila between the C—N and C—C bond form I think there is a great deal of simila there and it would be good not to fo about it. In some cases I think these situations may be treated as very simi

METZENBERG[7]: I should like to comm on a few properties of carbamyl ph phate synthetase, which might be ca a kinosynthase. These properties co well be interpreted as a concerted act Mammalian carbamyl phosphate syn tase catalyzes the reaction in figure

Now the ammonium ion concentration quired, if all these materials are pre in the form of sodium salts, is comple out of line with the concentration kn to occur in mammals. For example get the optimum synthesis of carba phosphate ~0.02 *M* ammonium ions necessary. When potassium ions are ad to the extent of about 0.025 *M*, howe this system will use ammonium ion extremely low concentrations. The affi of the system for ATP is similarly affe by the presence of potassium.

It has been impossible to measure Michaelis constant for ammonium for this particular over-all reaction for rather simple reason that ammonium

[6] Fritz Lipmann, The Rockefeller Institut Medical Research.

[7] R. L. Metzenberg, University of Wisconsi

able to replace potassium; so ammon-
n ions appear to be acting in a dual
e, first as a substrate and second as a
stitute for potassium. However, this
zyme will also catalyze a release of
osphate in the absence of ammonia if
other components of the reaction are
sent. Of course, no carbamyl phosphate
produced. This particular partial reac-
n is catalyzed if manganese instead of
usual magnesium is present as the ac-
ating ion.

In this case we do not have to add am-
nium ions in order to measure the
action and, therefore, it is possible to
tain Michaelis constants of a sort. Un-
these conditions we can measure the
chaelis constant for acetyl—glutamate
the total absence of ammonia. It turns
that the Michaelis constant for acetyl—
utamate as determined by phosphate re-
se is altered some 200-fold by the pres-
ce of potassium; it may be said that the
tassium alters the binding of this com-
und as well as of ammonia and ATP
en the entire reaction is allowed to pro-
ed.

This enzyme is much more stable in the
sence of potassium and can be kept in
refrigerator with virtually no loss for a
ek. When sodium instead of potassium
present drastic losses occur overnight
the refrigerator. It seems probable that
tassium changes the configuration of
s enzyme in such a way that all the
bstrates are more tightly bound and, in
dition, the stability of the enzyme is in-
enced. I want to mention that this work
s done in collaboration with Dr. M.
rshall and Dr. P. P. Cohen.

COHN[8]: Dr. Buchanan, did you use one
ner criterion to determine whether you
ve this concerted reaction or consecutive
actions; namely, the exchange of O^{18} in
e phosphate with that in the glycine in
e absence of ADP? Does it occur in the
sence of ADP? In the reaction Dr. Hager
entioned of CoA and succinate and ATP
ere is such an exchange without addition
ADP. I will say that, in the latter reac-
n, D. R. Sanadi and we too have found
senolysis occurring without ADP. Of
urse the enzyme is not sufficiently pure
us to be certain that there is not a
ce of ADP in the preparation, but as far as we know, there is arsenolysis, and also exchange between succinate, O^{18}, and phosphate without the presence of ADP, which would argue against a concerted reaction in that case.

BUCHANAN: Dr. Hartman, since you have been doing this work, it would be more appropriate for you to reply to Dr. Cohn's question.

HARTMAN: We have not done that experiment. It might be interesting to look into it. The fact that we do get stoichiometric transfer of oxygen, that is, 1 mole of oxygen from the phosphate to the carboxyl group of glycine formed, I think would at least eliminate a continuous exchange reaction.

COHN: In the succinyl—CoA reaction there is also only one oxygen from the phosphate transferred to the carboxyl group of succinate. This is a matter of rate, whether the equilibrium goes back and forth rapidly, but does not eliminate the possibility of observing such an exchange reaction under the proper conditions.

HARTMAN: In connection with Dr. Handler's observations on the azaserine inhibition, in the reaction in which 5-phosphoribosyl pyrophosphate is reacted with glutamine to form 5-phosphoribosylamine, we have found a very similar situation. Azaserine and another analog of glutamine, DON, will react with the enzyme in an apparently covalent fashion, and this reaction is very markedly stimulated in the presence of the substrate, 5-phosphoribosylpyrophosphate.

In regard to Dr. Metzenberg's comment concerning the effect of monovalent cations, one of the reactions of purine biosynthesis, the cyclization of formylglycinamidine ribonucleotide to 5-aminoimidazole ribonucleotide, exhibits an interesting dependence on monovalent cations. The enzyme involved here is stabilized by high levels of potassium and requires for activity the presence of a monovalent cation, either potassium, ammonium, or rubidium; whereas lithium, sodium, or cesium are completely inactive.

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[8] Mildred Cohn, Washington University, St. Louis.

SHAPIRA[9]: I think most organic chemists are quite aware that (and it is a possible pitfall in over-interpretation of *pK*'s, say, of ammonium ions) sometimes a very illogical reaction can proceed when two groups are in proximity. One example of this, which Drs. D. G. Doherty, J. X. Khym, and I studied is the rearrangement of aminoisothiuronium compounds, for example, 2-aminoethylisothiuronium bromide hydrobromide. This compound was isolated as a disalt. Now at *p*H 2, where it is illogical to assume that there can be a reasonable concentration of NH_2 (un-ionized amino group), this compound proceeds to cyclize and to liberate ammonium ions. The only reason this reaction goes at all is the close proximity of the two groups, and this means that, even if there is an infinitesimal amount of NH_2 present, the reaction proceeds immediately. So I doubt that you would predict that this reaction would occur at all based entirely on the *pK* of this amino group.

GREENBERG[10]: In the reaction of ribosylamine phosphate with glycine to form glycine ribonucleotide, there is one rather obvious difference between this reaction and some of the others, and that is that it is so freely reversible. This always bothered me. I wonder whether part of this free reversibility, if I may use this term loosely, is attributable to the nature of the amine. Is it possible that ribosylamine is an equilibrium mixture so that there is an imino compound formed rather than an amine?

BUCHANAN: It would be hard to say. It is not really fair to pick this one reaction out as being more freely reversible than any of the others. All the other reactions catalyzed by enzymes designated as kinosynthases are freely reversible.

GREENBERG: It was also my impression that it was very difficult to show that these other reactions were reversible at the beginning and that it is related to the order of magnitude of the equilibrium constant.

BUCHANAN: No, it is just a matter of technique. As better methods have become available it has been possible to demonstrate easily the reversibility of these other reactions.

GREENBERG: What is the equilibr constant of this reaction, say, compare glutamine synthetase?

BUCHANAN: We do not know. Bec of the destruction of the phosphoribo amine by our enzyme, which is only p ally purified, it is difficult really to de mine an equilibrium constant on the cinamide ribonucleotide synthase react

TODD[11]: What I have to say is re quite simple. It may not be a very hel contribution to the discussion but it app directly to what Dr. Lipmann was say He suggested that perhaps we should th of C—C and C—N bond formation being similar. All I would say is tha phosphatic intermediates are involved t are not merely similar—they are, in f identical reactions. It is merely a cas using the alkylating property of a p phate rather than its phosphorylating p erty. Two examples already mentione the discussion show this up very pla indeed.

They also indicate, I would suggest, PP is often used in biological reaction this type. This is clearly illustrated in case of the thiamine synthesis. The rimidine pyrophosphate is, of course, eq alent to a benzyl pyrophosphate, and i acts with the tertiary nitrogen of the t zole to give thiamine and PP, since f esterified benzyl and allyl compounds attacked by nucleophiles preferentially carbon to give a C—N linkage with ex sion of phosphate. In the laboratory make such reactions go well, it is usefu use a very strong acid like diphenylp phoric acid rather than phosphoric i to make the benzyl ester. I would sug that the PP is, indeed, in the enzyme tems simply because pyrophosphoric is a much stronger acid than phosph i.e., it will have a more stable anion therefore it will favor the alkylation r tion. The same kind of argument ca applied to the PP's that Dr. Lynen f are used in his biosynthetic reactions. virtue of pyrophosphoric acid is, accor to this view, simply its greater stren

[9] Raymond Shapira, Emory University.
[10] G. R. Greenberg, University of Michiga
[11] Alexander Todd, University Chemical La tory, Cambridge, England.

he short point is that C—C and C—N ond formation are clearly shown by the xamples we have had to be simply as- ects of one and the same reaction.

LIPMANN: I see that point very well. I ad not seen it before, but there is this ility of PP to disappear from the equili- ium, which I think in many cases is ally important in the biological system, ecause in many cases the reaction is more vored thermodynamically in the opposite rection and if you had phosphate instead PP you would push it all the way back.

TODD: Yes, there may be these addi- onal factors in biological systems. I erely wanted to make the point that om a straightforward chemical point of ew it is rational to use PP instead of osphate.

GREENBERG: Dr. Buchanan, you pointed it very briefly that the 10-formyl deriva- ve of folic acid will formylate imidazole rboxamide, but you must have the meth- yl compound to formylate glycine ribo- ucleotide. Do you presume that in one se a reversible system and in the other se an irreversible system is involved? r is this related to the difference in *pK* the two amino groups, or do you have me other explanation?

BUCHANAN: No, we do not, but it is ssible that the *pK* of the amino group ould be the important factor.

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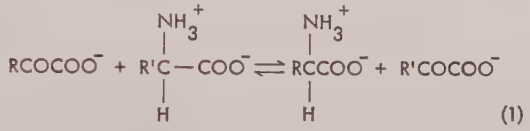
he Mechanism of the Transamination Reaction

ESMOND E. SNELL AND W. TERRY JENKINS
Department of Biochemistry, University of California, Berkeley

Enzymic transamination between amino nd keto acids was discovered by Braunein and Kritzmann in 1937. Subsequent udies have shown it to be of very wide cope, involving as substrates most of the mino acids, including glutamine and asaragine, and the corresponding keto cids. The over-all reaction is shown in quation (1) (fig. 1). The activity, speciity, and number of distinct transamases vary from one organism to another nd perhaps from one tissue to another a single organism. Although L-α-amino cids are the most common amino donors, ansaminases that operate with β- and γmino acids, certain D-amino acids, and ubstrates containing no carboxyl groups so are known. The scope and signifince of the enzymic reaction have been viewed frequently (Braunstein, '47; ohen, '51; Meister, '55, '57) and will not e considered further here.

The mechanism of the transamination action and of related reactions that require pyridoxal phosphate as coenzyme can be considered in two stages: (1) the role played by the chemically defined coenzyme and the features of its structure that make this role possible, and (2) the contributory role played by the chemically ill-defined apoenzymes in catalysis of these reactions. We may consider these topics in turn.



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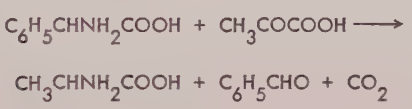

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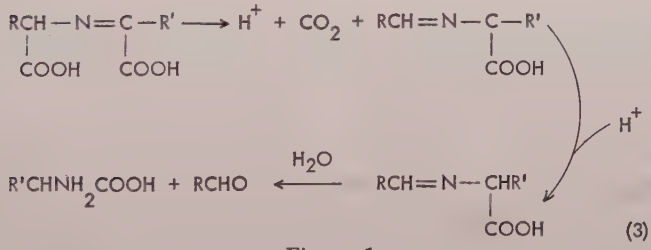


(3)

Figure 1

Chemical models of the transamination reaction

Early models. The role played by the coenzyme was first revealed through study of chemical models of the transamination reaction. Herbst and Engel ('34) studied a reaction between α-amino and α-keto acids that is similar to enzymic transamination. When a mixture of α-aminophenylacetic and pyruvic acids was boiled in water, benzaldehyde, CO_2, and alanine were formed in approximately equimolar amounts [eq. (2)]. The reaction was unaffected by acid but was inhibited by suf-

ficient alkali to cause salt formation. A primary amino group was prerequisite for these reactions; subsequent studies with amino acids labeled with deuterium on the α-carbon atom showed that this was not lost during the reaction but was largely retained in the benzaldehyde formed. In D_2O, the alanine formed contained deuterium in both the α and β positions. Brewer and Herbst ('41) suggested the reactions occurred by isomerization of an initially formed Schiff base, accompanied by decarboxylation and followed by hydrolysis, as shown in equation (3).

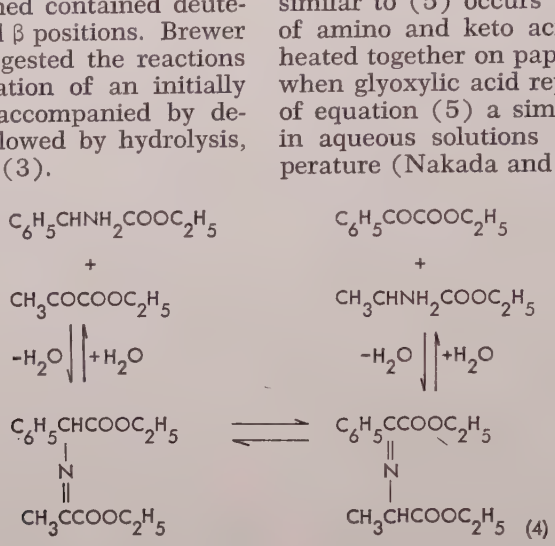


Figure 2

When the carboxyl groups of both reactants were blocked by esterification and the reaction was carried out in an anhydrous solvent, transamination but not decarboxylation occurred; an analogous scheme [eq. (4)] was proposed to explain this reaction (see fig. 2). With the discovery of enzymic transamination, an analogous series of reactions involving free amino and keto acids was assumed to occur at the enzyme surface. However, because the α-hydrogen of the react amino acid was labilized during this re tion (Konikova *et al.*, '42), Braunstein a his group proposed a slight modification the mechanism of Herbst for the enzy reaction. This is shown in equation ((fig. 3; see Braunstein, '47). A react similar to (5) occurs with a wide vari of amino and keto acids when these heated together on paper (Giri *et al.*, '5 when glyoxylic acid replaces the keto a of equation (5) a similar reaction occ in aqueous solutions even at room te perature (Nakada and Weinhouse, '53)

Models involving pyridoxal. These ea postulates concerning mechanism prece discovery of pyridoxal and pyridoxami Even before these compounds beca available in pure form by synthesis, it v evident that pyridoxal, in the presence amino acids, was transformed in part a compound possessing biological pr erties characteristic of pyridoxamine. soon as the pure compounds became av able it was shown by isolation of all pr

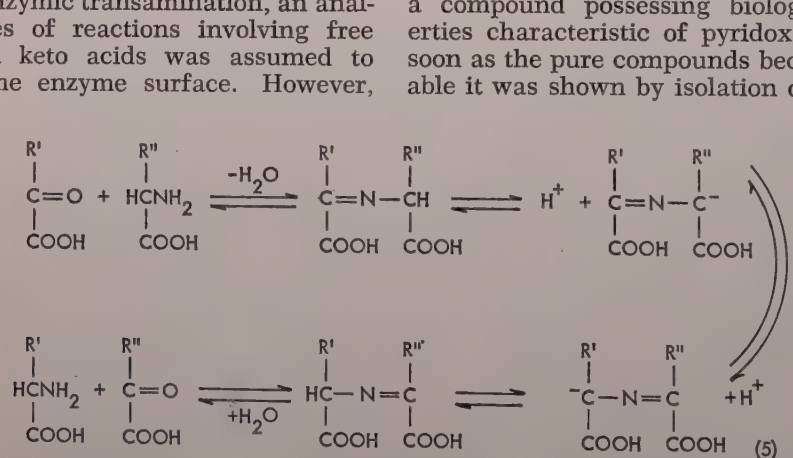


Figure 3

$$\text{Glutamate} + \text{pyridoxal} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{pyridoxamine} \quad (6a)$$

$$\text{Amino acid}_1 + \text{pyridoxal} \rightleftharpoons \text{pyridoxamine} + \text{keto acid}_1 \quad (6b)$$

$$\text{Keto acid}_2 + \text{pyridoxamine} \rightleftharpoons \text{pyridoxal} + \text{amino acid}_2 \quad (6c)$$

$$\text{Sum: amino acid}_1 + \text{keto acid}_2 \rightleftharpoons \text{amino acid}_2 + \text{keto acid}_1 \quad (6)$$

ıcts that reaction (6a) occurred in neutral ıqueous solution and was fully reversible Snell, '45). Microbiological assays of ıeated reaction mixtures showed the reıction occurred over a wide *p*H range and vith a wide variety of amino acids. It was ecognized that summation of two such reersible reactions, as shown in equations to include intermediate Schiff base formation and transamination with pyridoxal (PyCHO) or pyridoxamine ($PyCH_2NH_2$), equation (6b) becomes (7b) and (6c) becomes (7a) (fig. 4). The same formulation suffices to explain the enzymic reaction, where PyCHO now represents a pyridoxal phosphate protein and $PyCH_2NH_2$ the

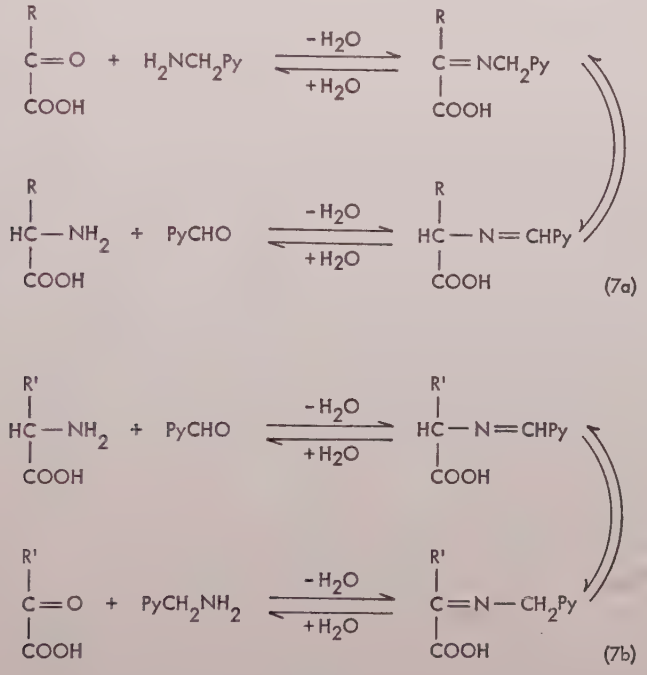


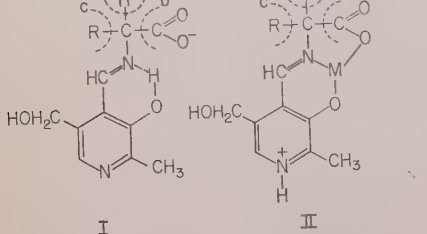
Figure 4

(6b) and (6c), would give a result identical with that of enzymic transamination, in which pyridoxal and pyridoxamine would be required only as catalysts (Snell, 45). These experiments were the basis for our early suggestion that pyridoxal and pyridoxamine might be involved in enzymic transamination (Snell, '44) and led directly to recognition of the essential role of pyridoxal phosphate in enzymic transamination (Schlenk and Snell, '45; Green *et al.*, '45; Lichstein *et al.*, '45). By extension of the Herbst-Braunstein formulation corresponding pyridoxamine phosphate protein (Schlenk and Fisher, '47), and the vitamin B_6 derivatives catalyze the reaction by serving alternately as amino group acceptor and donor.

Until recently, this scheme for the enzymic reaction was supported chiefly by analogy with the nonenzymic reaction that suggested it. It will be useful, therefore, to examine this nonenzymic reaction more closely for the light it can throw on the more intimate details of the catalytic process. Reference to a recent review (Snell,

'58) provides further documentation for matters considered only briefly here.

The formation of the postulated imines between pyridoxal or its phosphate and amino acids in dilute aqueous solutions can be detected by the marked increase in absorption that occurs in the 345- to 430-mμ region of the spectrum when solutions of the two reactants are mixed. The reaction is freely reversible and approaches



I II

Figure 5

completion only with relatively high concentrations of amino compound. Pyridoxal phosphate cannot form an internal hemiacetal and hence forms such imines to an even greater extent than pyridoxal. In the neutral *p*H range, these imines appear to be hydrogen-bonded structures such as I, figure 5 (Metzler, '57). The corresponding imines formed between pyridoxamine and keto acids have not yet been studied.

As shown in table 1, the rate of non enzymic transamination was increased a much as 20-fold by addition of appropriat metal ions (Metzler and Snell, '52). B analogy with the well-known chelate com pounds formed from salicylaldehyde, meta ions, and amines, the metal was assume to promote the reaction through forma tion of chelate complexes of the nature o II, figure 1, which contained vitamin amino acid, and metal ion (Snell, '53 Metzler, Ikawa, and Snell, '54). Severa complexes of this nature have been isolate (e.g., *ibid.;* Metzler, Longenecker, an Snell, '54; Baddiley, '52). Their forma tion has also been detected spectrophoto metrically (Eichhorn and Dawes, '54) an by paper electrophoresis of ternary mix tures of pyridoxal, alanine, and metal ion (Fasella *et al.*, '57).

The course of a typical metal ion cata lyzed, nonenzymic transamination reactio between pyridoxal and leucine is show in figure 6. Equilibrium is approache from either direction at similar rates; th equilibrium position for this amino aci lies at approximately 50% reaction. Mos amino acids react under these condition at similar rates, but β-substituted amin acids (e.g., valine, isoleucine) react muc more slowly, and in some instances impor tant side reactions prevent uncomplicate

TABLE 1[a]

The comparative activities of metal ions in catalysis of transamination between pyridoxamine and α-ketoglutarate[b]

Metal ion[c]	m*M*	Optimum *p*H	Metal ion (m*M*) × heating time (min)		
			2	4	8
			Pyridoxal formed, m*M*		
Ga(III)	0.125	4.3	2.9	3.9	4.7
Cu(II)	0.125	4.8	2.6	3.4	4.3
Al(III)	0.125	4.8	2.2	2.9	3.5
Fe(II)	0.125	4.8	1.5	2.1	3.0
Fe(III)	0.25	4.8	1.3	1.8	2.6
Zn(II)	0.5	7.0	1.1	1.7	2.5
In(III)	0.5	4.3	0.8	1.4	2.0
Ni(II)	0.5	8.0	0.7	1.3	1.9
Co(II)	0.5	7.0	0.5	0.9	1.1
Sc(III)	0.5	6.0	0.4	0.7	1.0
None	—	5.0	0.2 in 16 min	0.4 in 32 min	0.7 in 64 min

[a] From Longenecker and Snell ('57). Less-complete data showing effects of versene and metal-low reagents are given by Metzler and Snell ('52).

[b] Unbuffered reaction mixtures contained 10 m*M* pyridoxamine, 10 m*M* α-ketoglutarate, metal ion as indicated, and were heated at 100°C.

[c] Slight activity in the following order, was expressed by Sm(III) > Pt(IV) > Nd(III) > Cd(II) > Cr(II) > Mn(II) > Mg(II).

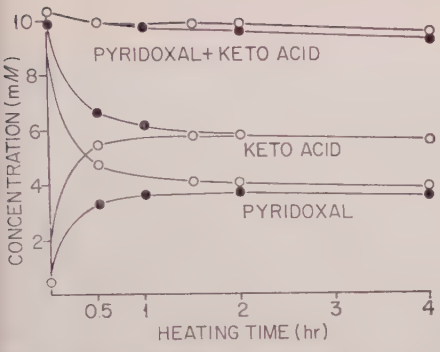


Fig. 6 Nonenzymic transamination at 100°C. etween leucine and pyridoxal. Alum (0.001 *M*);), between 0.01 *M* pyridoxamine and 0.01 *M* etoisocaproate; ●, between 0.01 *M* pyridoxal and .01 *M* leucine. From Metzler and Snell ('52).

bservation of the transamination reaction Metzler and Snell, '52; Snell, '58).

Occurrence of such rapid, nonenzymic ransamination reactions made possible a airly exact delineation of the structural eatures of the pyridoxal molecule required or the reaction (Metzler, Ikawa, and Snell, 54; Metzler, Olivard, and Snell, '54; Ikawa nd Snell, '54; Snell, '58). Results of this nvestigation, shown in table 2, demonstrate that the minimum structural features required for transamination are a ormyl group *ortho* or *para* to a strongly electronegative group, such as the heterocyclic nitrogen atom of pyridoxal, and *ortho* to a free phenolic group. These minimum requirements are supplied by 2-formyl-3-hydroxypyridine and by pyridoxal, but not by isopyridoxal, 3-nitrosalicylaldehyde, or other closely related compounds. The 5-hydroxymethyl group of pyridoxal, though not required for the nonenzymic reaction is, of course, required for coenzyme formation, and hence for the enzymic reactions catalyzed by pyridoxal phosphate.

Each of the catalytically effective aldehydes can form a Schiff base with amino acids that can be stabilized by hydrogen bonding as in I, figure 5, or by chelate formation, as in II. In each of these complexes, a conjugated system of double bonds extends from the α-carbon of the amino acid to the electron-attracting group of the complex, thus permitting electron withdrawal from, and consequent weakening of, bonds a, b, and c of the complex. It is this weakening of these bonds that constitutes "activation" of the amino acid and permits catalysis by pyridoxal of the several reactions of amino acids, including transamination, that are promoted by this vitamin, its 5-phosphate, and enzymes that contain the latter (Metzler, Ikawa, and

TABLE 2

Relation of structure to ability of aldehydes to react with glutamate to form α-ketoglutarate[a]

Aldehyde	Ketoglutarate formed[b]	
	+ Alum	− Alum
Pyridoxal	+++	−
5-Deoxypyridoxal	+++	−
Pyridoxal phosphate	+++	−
ω-Methyl pyridoxal	+++	−
2-Formyl-3-hydroxypyridine	+++	−
4-Nitrosalicylaldehyde	+++	−
6-Nitrosalicylaldehyde	+++	+
Glyoxylic acid	+++	
Isopyridoxal(2-methyl-3-hydroxy-4-hydroxymethyl-5-formylpyridine)	−	−
3,*O*-Methylpyridoxal	−	−
Salicylaldehyde	−	−
p-Nitrobenzaldehyde	±	±
5-Nitrosalicylaldehyde	−	
3,5-Dinitrosalicylaldehyde	−	
4-Carboxysalicylaldehyde	−	
2,4-Dihydroxybenzaldehyde	−	
2-Hydroxy-4-chlorobenzaldehyde	−	

[a] Glutamate (10 m*M*), aldehyde (10 m*M*), 100°C. for 30 minutes, *p*H 50, ± 1 m*M* alum. Adapted from Snell ('58).

[b] − Signifies no reaction; ±, barely detectable reaction; +, and +++, increasing degrees of reaction.

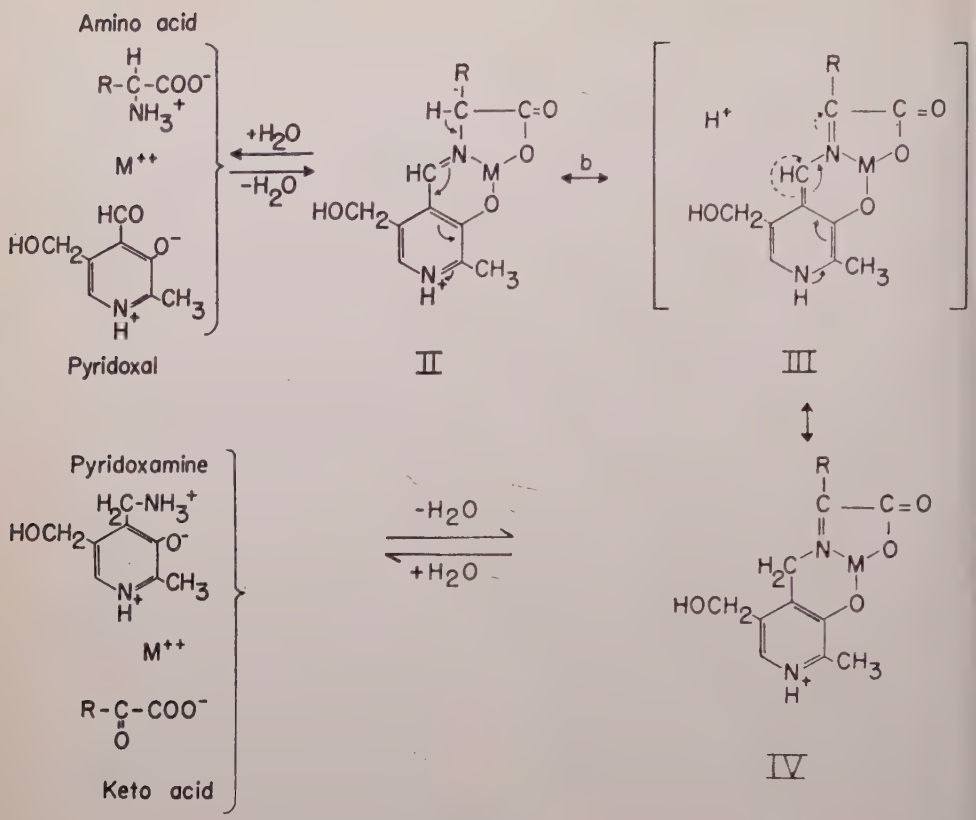


Figure 7

Snell, '54; Braunstein and Shemyakin, '53; Snell, '53, '58).

The picture of the nonenzymic transamination reaction that arises from these studies is shown in figure 7, which depicts in more detail what is believed to occur during reaction (7b). Electron withdrawal from the bond to the α-hydrogen atom labilizes the proton and permits shift of the double bond to yield the isomeric Schiff base of pyridoxamine and a keto acid that in aqueous solution is in equilibrium with its components. In the reverse reaction, it is a proton of the methylene group in the 4 position of pyridoxamine that is labilized in this same fashion. Once the primary act of catalysis, labilization of the hydrogen atom is achieved, II, III, and IV (fig. 7) can be considered as resonating forms of the same structure. The close relation of the reaction to racemization is apparent.

The role of metal ions in nonenzym transamination. Although the rate of n enzymic transamination is increased ma edly by appropriate metal ions (see ta 1), some reaction occurs even thou metal-low reagents are used in the prese of ethylenediaminetetraacetate. Un these conditions the reaction may proc via the hydrogen-bonded intermediat (fig. 5). Addition of metal ions perm formation of intermediates of the nat of II (fig. 5).

In the absence of definitive informat concerning structure of each reactive s cies, we can only speculate concern reasons for the increased rate. Throu chelation with the amino acid, metal i may both facilitate formation of the Sc base intermediates and contribute to th stability. Second, if the structures of ure 5 are representative of those that oc in neutral solutions, it will be appar

TABLE 3

Comparison of enzymic, pyridoxal-catalyzed, and uncatalyzed transamination between keto and amino acids[a]

Nonenzymic transamination		Enzymic transamination
Uncatalyzed	Pyridoxal-metal catalyzed	
1. Proceeds slowly at 100°C.	Proceeds rapidly at 100°C., slowly at room temperature.	Proceeds rapidly at room temperature.
2. Most rapid in acid solution; completely inhibited at neutral or alkaline reaction.	Optimal *p*H 4.3–8.0, depending upon metal ion, inhibited below *p*H 3.0 or above 9.5.	Optimal (glutamic–aspartic) *p*H 7.5–8.2; complete inhibition below p*H* 3.5 and above 9.5.
3. Irreversible owing to decarboxylation of amino acid.	Completely reversible; involves no decarboxylation.	Completely reversible, involves no decarboxylation.
4. Uncharged COOH group detached as CO_2; α-hydrogen of original amino acid retained in resulting aldehyde; α position of new amino acid filled by a proton from medium.	Undetermined, but reaction requires α-hydrogen[b] and racemization occurs at high *p*H; in all probability same therefore as enzymic reaction.	Rapid exchange of α-hydrogen of reactant amino acid with water; α-hydrogen of product amino acid filled by protons from aqueous medium.
5. No specificity for configuration. With optically active original amino acid, the newly formed amino acid is racemic.	Partial stereochemical specificity. If initial amino acid is L, product amino acid contains an excess of L isomer. Converse is also true.	Complete stereochemical specificity. Reactant L-amino acid yields product L-amino acid; reactant D-amino acid yields product D-amino acid.

[a] Adapted and extended from Braunstein ('47).

[b] Slow irreversible transamination occurs between α-substituted amino acids and pyridoxal to yield pyridoxamine (in part), CO_2, and the aldehyde or amine corresponding to the decarboxylated amino acid (Kalyankar and Snell, '58 and unpublished). The reaction appears analogous to that of Herbst.

that the quaternary nitrogen of the chelate structure is more highly electronegative than the tertiary nitrogen of the hydrogen-bonded structure and hence should be more effective in displacing electrons through the conjugate system. The bonded carboxyl group of the chelated structure can also contribute to this labilization much more than the carboxylate ion of I (fig. 5), and finally, the electronegative metal ion may itself contribute to this displacement. Third, because of the planar structure of chelate compounds, the metal ion serves to hold the system of conjugated double bonds in the planar configuration essential for the postulated electron shifts.

These three possible roles for the metal ion are similar to those generally ascribed to the protein moiety of holoenzymes (Hoare and Snell, '58; Snell, '58). In the first role, the metal ion facilitates interaction between "substrate" (amino acid) and the active site of the "enzyme" (pyridoxal); in the second role, the metal ion contributes to those features of the structure of the intermediate that lead to the desired polarization; and in the third role, it stabilizes a necessary configuration of the transitional form sufficiently to increase the chances for reaction. Whether the metal ions of the nonenzymic systems serve solely as models for the apoenzyme moiety of vitamin B_6 enzymes, or whether they are required for certain of the enzymic reactions is a question that can be resolved satisfactorily only by analyzing purified enzymes. No one of these postulated roles for metal ions in the model systems seems to require metal ions exclusively; and, as originally pointed out (Metzler, Ikawa, and Snell, '54), it is quite possible that the role played by metal ions in the nonenzymic reactions may be served much more efficiently by protein alone in the corresponding enzymic reactions. Several highly purified vitamin B_6 enzymes, including the glutamic–aspartic transaminase of pig heart (Jenkins and Sizer, '59), have now been found not to contain stoichiometric quantities of metal ions. The report that the glutamic–aspartic transaminase of green beans requires Fe^{++} for activity (Patwardhan, '58) thus needs further investigation.

Comparison of nonenzymic and enzym transamination. A comparison of u catalyzed nonenzymic transamination r actions with corresponding reactions cat lyzed by pyridoxal and metal salts or h enzymes is presented in table 3. The pyr doxal–metal ion-catalyzed reaction is stri ingly similar to the enzymic reaction an differs significantly from the uncatalyze reaction described by Herbst ('44). Th partial optical specificity of the pyridoxa metal ion-catalyzed reaction deserves com ment since the transition form IV (fig. 7 is not asymmetric. However, the met ion of this compound has unoccupied c ordination positions, and by binding molecule of an optically active reactan the complex becomes optically active. I the conversion to the isomeric Schiff bas analogous to II (fig. 7), assumption one of the two possible enantiomorph configurations will be favored. In the cas investigated, the optical specificity is the same direction as that exhibited by th enzymic reaction (Longenecker and Sne '56).

The mechanism of the enzymic transamination reaction

Two principal types of mechanisms f the enzymic transamination reaction ha been proposed. In the ternary mechanism it was assumed that interaction occurr simultaneously at a single enzymic si among all three reactants (amino dono coenzyme, and amino group acceptor Such ternary hypotheses do not satisfa torily explain (1) the equivalent and ma imal activation of apotransaminases pyridoxal phosphate and pyridoxami phosphate (Meister *et al.*, '54), (2) the served transamination from an amino ac to the keto acid of corresponding stru ture (Nisonoff *et al.*, '54; Jenkins a Sizer, '59), (3) the formation of keto aci and a pyridoxamine phosphate enzym during the reaction of amino acids w substrate amounts of purified transa nase (Jenkins and Sizer, '57), or (4) existence of two forms of the transamina which are readily interconverted by ad tion of either amino or keto acid (Jenk and Sizer, '57). Certain data concerni the kinetics of the reaction were erro ously interpreted to support such a me

ism; these data are, however, equally ll explained by the binary mechanisms nsidered below (see Longenecker and ell, '56). Such ternary hypotheses must, erefore, be abandoned.

Each of the observations is adequately plained by the *binary* hypothesis, accord- g to which enzymic transamination, like e pyridoxal-catalyzed model systems, is e sum of two separate binary reactions the sense illustrated by equations (6b) d (6c) and in figure 8. In principle,

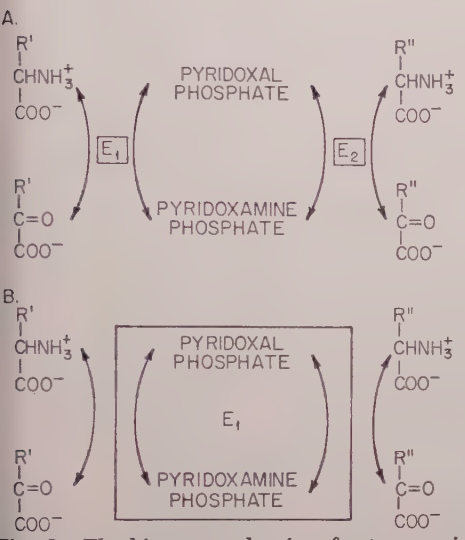


Fig. 8 The binary mechanism for transamina- n. In A, pyridoxal and pyridoxamine phos- ates couple transamination reactions catalyzed two separate transaminases; in B, two forms the same enzyme catalyze the reaction. Only echanism B has so far been observed.

ese two binary reactions could be cata- zed by two distinct enzymes, one specific r an amino acid and pyridoxal phosphate substrates, the other for a keto acid and ridoxamine phosphate, as shown in fig- e 8A. This system has not so far been served and is inconsistent with the prop- ties of purified transaminases so far udied, in which the reaction between ridoxal phosphate and apoenzyme is very w and that with pyridoxamine phos- ate even slower (Meister *et al.*, '54). rthermore, prolonged dialysis of the glu- mic–aspartic holotransaminase does not move the catalytically essential amounts pyridoxal or pyridoxamine phosphates en in reconstituted preparations, and ese prosthetic groups are not readily dis- placed from the apoenzyme by coenzyme analogs (Meister *et al.*, '54; Snell, '58). In addition, no unequivocal demonstration with purified enzymes that transamination occurs between amino acids and pyridoxal phosphate or between pyridoxamine phosphate and keto acids has so far appeared. However, occasional reports of such transamination reactions in crude extracts (Meister *et al.*, '51; Beechey and Happold, '57) need further investigation to permit assessment of the possible significance of such reactions, which would permit coupling between transaminases at the coenzyme level.

The over-all mechanism shown in figure 8B is certainly that which holds for the glutamic–aspartic transaminase. It is the sum of two binary reactions between an amino acid and a pyridoxal phosphate enzyme and between a keto acid and a pyridoxamine phosphate enzyme. It is the over-all mechanism suggested in 1947 by Schlenk and Fisher on the basis of model experiments of Snell ('45); its proof, however, was possible only after isolation of the transaminase in essentially pure form and detailed examination of its interaction with its substrates. We may conclude by considering the nature of this proof and certain of its implications.

The purification procedure of Jenkins *et al.* ('59) was based on the observation that maleate buffer markedly enhanced the stabilizing effect of ketoglutarate (Mason, '57) and permitted a high degree of purification of the glutamate–aspartic transaminase (holoenzyme) in good yield by heat denaturation of impurities (table 4). Further purification by ammonium sulfate fractionation and chromatography on calcium phosphate yielded a preparation 80–85% pure as indicated by ultracentrifugal, electrophoretic, and spectroscopic studies. Sedimentation and diffusion gave a molecular weight of 110,000. As prepared in the presence of α-ketoglutarate, the enzyme contains 2 moles of pyridoxal phosphate per mole of protein. This is liberated by heat denaturation or by addition of acid or alkali.

A striking feature of the pyridoxal enzyme is its behavior as a *p*H indicator (*pK* 6.2) with a bright yellow acidic form, λ_{max} 430 mμ, and a colorless basic form,

TABLE 4

Major steps in preparation of glutamic–aspartic transaminase in its pyridoxal and pyridoxamine forms

Step	Procedure
1	Disperse 20 lb of minced pig heart ventricles in 1½ volumes of 0.05 *M* maleate buffer, *p*H 6.0, for 30 seconds in a blender.
2	Heat to 75°C., adding 0.01 mole of ketoglutarate when the temperatures reaches 60°. Maintain at 75°C. for 20 minutes, then cool in an ice bath.
3	Strain through muslin overnight. Collect the material soluble in 50% but insoluble in 67% ammonium sulfate. Dissolve in 0.2 *M* maleate buffer, *p*H 6.0, and dialyze against water.
4	Chromatograph on a hydroxylapatite (Tiselius) column. Reprecipitation of the first yellow band with ammonium sulfate gives the pyridoxal form of the transaminase.
5	Add excess glutamate and Tris-formate buffer, *p*H 8.3. Pass through Dowex-1-formate column and dialyze the pooled enzyme fractions. Column removes ketoglutarate as formed, then excess glutamate and yields the pyridoxamine form of the transaminase.

λ_{max} 362 mμ. A single isosbestic point is obtained as the *p*H is varied, suggesting that the *pK* obtained spectrophotometrically represents the dissociation constant of a single proton (fig. 9). The yellow

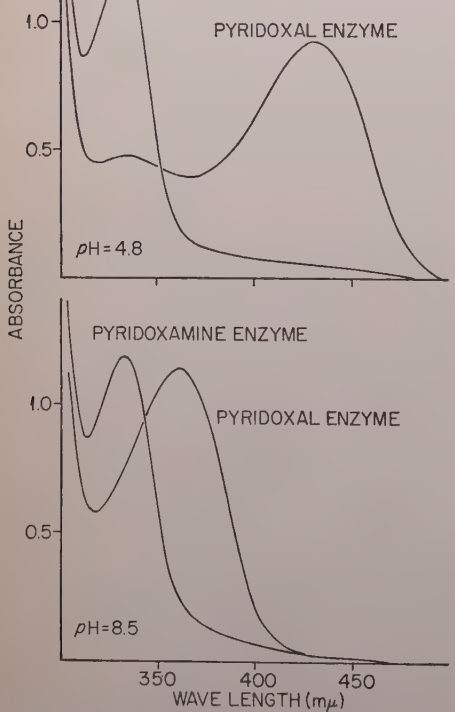


Fig. 9 Spectra of the pyridoxal and pyridoxamine forms of the glutamic–aspartic transaminase at two *p*H values. Buffers: 0.15 *M* acetate *p*H 4.8 and 0.1 *M* pyrophosphate *p*H 8.5.

form of the enzyme seems to be catal cally inert, for the velocity of transami tion increases in inverse proportion to amount of this structure present (Jenk and Sizer, '59).

This change from yellow to colorl with increase in *p*H is a property not pyridoxal phosphate but of the pyrido phosphate imines (Metzler, '57). On and other grounds, Jenkins and Sizer ('5 suggested that the pyridoxal phosph was bound through its formyl group to amino group on the enzyme. The mu lower *pK* for the enzyme than for pyridoxal phosphate imines may indic that the heterocyclic nitrogen of the p doxal is protonated at all times. Fisc *et al.* ('58) showed similar binding of p doxal phosphate for phosphorylase. In c trast to the behavior of phosphoryla where the formyl group of pyridoxal ph phate is apparently not required for zymic activity, reduction of the pyrido form of the transaminase with sodi borohydride yields an inactive derivati

Addition of glutamate to the pyrido transaminase causes an increase in abso ance at 332 mμ and a concomitant crease at 362 mμ, both of which are pendent on the glutamate concentrat (Jenkins and Sizer, '57, and unpublishe Ketoglutarate is formed in the reacti and an equivalent amount of enzy bound pyridoxal phosphate disappe The latter could be accounted for as zyme-bound pyridoxamine phosphate. form of the enzyme may be prepared

the pyridoxal phosphate form by pass- g a mixture of glutamate and pyridoxal zyme through a Dowex-1-formate col- ın since ketoglutarate is continually ad- rbed before the glutamate (table 4). The ridoxamine enzyme has a λ_{max} at 332 ı over a wide range of *p*H values. Addi- n of trace amounts of ketoglutarate or aloacetate instantly reconverts it to the ridoxal enzyme. In contrast to the pyri- xal form of the enzyme, the pyridox- ıine form is not inactivated by treatment th borohydride. These facts, then, estab- h the shuttle mechanism of the type own in equations (6) and (7) (fig. 4) d figure 8B as the over-all mechanism r the glutamic–aspartic transaminase.

This mechanism carries with it certain ıportant implications.

(1) There is no specificity for amino id–keto acid substrate pairs, but only for dividual amino and keto acids. Thus, if particular keto acid, e.g., α-ketoisovaleric id, serves as an amino group acceptor ɔm a particular "pyridoxamine" enzyme, en all amino acid substrates (i.e., those at will react with the pyridoxal form of at enzyme) will transaminate with keto- ɔvaleric acid to form valine.

(2) Since both an amino acid and its alog keto acid are substrates as defined, ınsamination will occur from one to the ıer and may be observed either as an parent inhibition at the equilibrium int (Nisonoff *et al.*, '53, '54), or as an change between labeled amino acid and e corresponding keto acid. Glutamate, r example, inhibits the glutamic-aspartic ınsaminase in this fashion by competing th reactant aspartate for reactant keto- ıtarate.

(3) Any amino acid–keto acid pair will rve to couple two transaminases if the ıino acid will react with the pyridoxal ɪm of one transaminase and the keto id with the pyridoxamine form of an- ıer transaminase. There is no reason to lieve, on this basis, that different trans- ıination reactions are coupled only rough glutamate–ketoglutarate.

(4) Since overlapping specificities for e substrate of a transaminase are quite mmon, more attention needs to be given specificity of assays. An assay based, r example, on transamination from leu- cine to ketovaline, might well measure more than one enzyme, just as transamination from radioactive glutamate to unlabeled ketoglutarate will be catalyzed by many different transaminases.

The availability of substantially pure glutamic–aspartic transaminase makes possible a variety of experiments to determine the nature of and equilibria between various possible intermediate complexes involved in transamination. Two such complexes have been formulated for both nonenzymic (fig. 7) and enzymic [eq. (7)] reactions. The latter may be described schematically in the terms in equation (8) (fig. 10) where E_1 and E_2 are forms of the

$$E_1 + S_1 \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightleftharpoons E_2 + S_2 \qquad (8)$$

$$\frac{(E_1)(S_1)}{(ES_1)} = K_{S_1}; \quad \frac{(E_2)(S_2)}{(ES_2)} = K_{S_2}; \quad \frac{ES_1}{ES_2} = K,$$

$$\frac{S_1}{\Delta E_1} = \frac{1}{E_t}\left[S_1 + \frac{K \cdot K_{S_1}}{\left(1 + K_{S_2/S_2}\right)}\right] \qquad (9)$$

Figure 10

enzyme, and ES_1 and ES_2 are possible enzyme—substrate complexes. If only one stable enzyme–substrate complex is formed $K = 1$.

Steady-state kinetics for this system require that, if the concentration of one substrate (S_2) is kept constant, the loss in E_1 is related to the concentration of the other substrate (S_1) by equation (9), where E_t is the total enzyme in all forms and ΔE_1 is the loss in E_1 caused by addition of S_1.

Increasing the ratio of glutamate to ketoglutarate or increasing their concentrations in constant ratio causes a decrease in the absorbance of the pyridoxal enzyme (E_1) at 362 mμ owing to the formation of enzyme–substrate intermediates and the pyridoxamine enzyme E_2. An increase in absorbance at about 330 mμ in the latter case must be attributable to one or both of the ES forms. No spectral evidence for two such forms is obtained, but this may reflect only a close similarity in absorption maxima, or a value of K such that only small amounts of one are present. If the

concentration of keto acid (S_2) is kept constant, the decrease in absorbance at 362 mμ may be taken as a measure of ΔE_1. If this is now measured as a function of the glutamate concentration (S_1), $K \cdot Ks_1/(1 + Ks_2/S_2)$ may be determined (fig. 11). From this the values of $K \cdot Ks_1$ and Ks_2 may also be derived since S_2 is known. The maximum change in absorbance (equivalent to E_t) derived graphically showed that virtually all of the absorption at 362 mμ was attributable to the pyridoxal enzy alone. For the glutamate–ketoglutar system at *p*H 9.8, the value of Ks_2 w 0.0017 *M* and that of $K \cdot Ks_1$ was 0.050 The ratio of $K \cdot Ks_1$ to Ks_2 was therefore (Jenkins and Sizer, unpublished). T high value suggests that only one int mediate (ES_1) appears in any substant concentration.

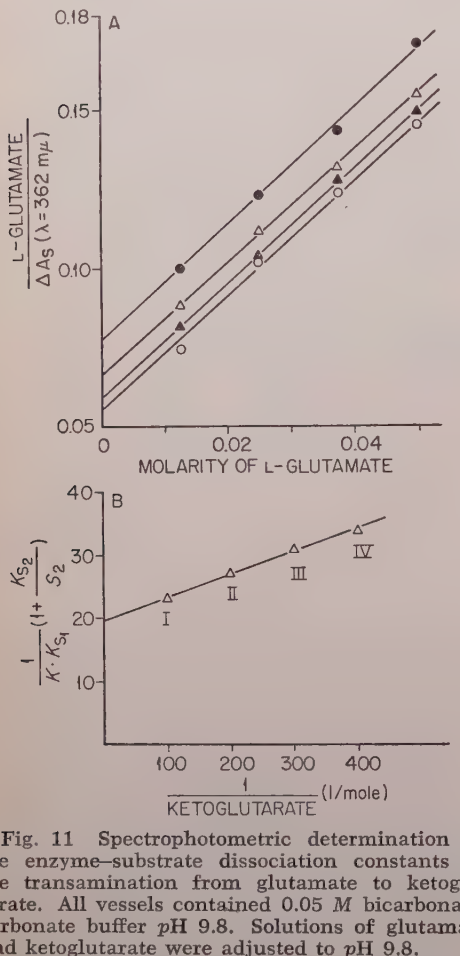


Fig. 11 Spectrophotometric determination of the enzyme–substrate dissociation constants in the transamination from glutamate to ketoglutarate. All vessels contained 0.05 *M* bicarbonate-carbonate buffer *p*H 9.8. Solutions of glutamate and ketoglutarate were adjusted to *p*H 9.8.

A. Effect of increasing the concentration of glutamate on the absorbance at 362 mμ at four different concentrations of ketoglutarate: I, 0.01 *M*; II, 0.005 *M*; III, 0.0033 *M*; IV, 0.0025 *M* from top to bottom.

B. The negative intercepts in figure 11A as a function of the concentration of ketoglutarate.

The rate of the reaction with glutam and ketoglutarate was too high to measu at these high levels of the enzyme. Th with alanine and similar compounds r usually considered substrates of this e zyme is extremely low and may be studi conveniently in the spectrophotometer sufficiently high levels of "substrate" a used. When the same analysis was appl to the alanine–pyruvate system at equil rium (*p*H 8.3), values of $K \cdot Ks_1$ and obtained were both very high (> 5 *M* their ratio was 160. The high concent tion of alanine required for measura complex formation is a reflection, course, of the substrate specificity of enzyme. This can be best expressed terms of the turnover numbers, and shown for several substrates in table Several amino acids react at rates t appear little higher than those with p doxal phosphate alone; leucine is rep sentative of substrates that apparently c not approach the reactive site of the zyme and hence react even slower than the nonenzymic reaction. The high s cificity of the enzyme for dicarbox

TABLE 5

Approximate turnover numbers for the interac of purified glutamic–aspartic transamina with various substrates

Reactants[a]	Turnove numbe
	min^{-1}
Glutamate-α-ketoglutarate	50,000
Aspartate-α-ketoglutarate	25,000
Alanine-pyridoxal enzyme	10
Methionine sulfoxide-pyridoxal enzyme	1
Methionine sulfone-pyridoxal enzyme	1
Glutamine-pyridoxal enzyme	1
Leucine-pyridoxal enzyme	0

[a] Physiological substrates were at satura concentrations; these cannot be achieved the substrate analogs, alanine, methionine su ide, etc., which were used at 1 *M* concentra

ino acids is very probably related to the ibition of the transaminases at low *p*H dicarboxylic acids. This inhibition re-ts from complex formation; with the ridoxal enzyme a decrease in absorbancy 362 mμ, accompanied by an increase ar 435 mμ, occurs (Jenkins *et al.*, '59; ison, '58). Such complex formation re-ls the occurrence near the prosthetic oup of enzymic sites that bind the di-rboxylic acids, thus facilitating their eraction with the prosthetic group.

The picture that emerges from these idies is of an enzyme of undefined struc-re with a firmly bound prosthetic group it can exist either as pyridoxal phos-ate or as pyridoxamine phosphate. The mer coenzyme is bound to the apoen-me by imine formation with an amino oup of the protein and by ionic linkages, illustrated schematically in figure 12. the imine linkage to protein to form one or more intermediates spectrally distinct from the initial enzyme and probably of the imine type postulated for the nonenzymic transamination reaction. Isomerization of the imine as a result of labilization of the α-hydrogen then occurs to yield a pyridoxamine phosphate enzyme and a keto acid. This labilization is possible because of the peculiar structure of the prosthetic group, as shown by the nonenzymic studies, and may be enhanced by appropriately placed ionic groupings on the protein moiety of the enzyme. The nature of the protein residues to which the formyl group and phosphate groups of the coenzyme are linked, of the intermediate enzyme—substrate complexes, and of the way in which the protein enhances reactivity of the coenzyme and limits its availability to a few

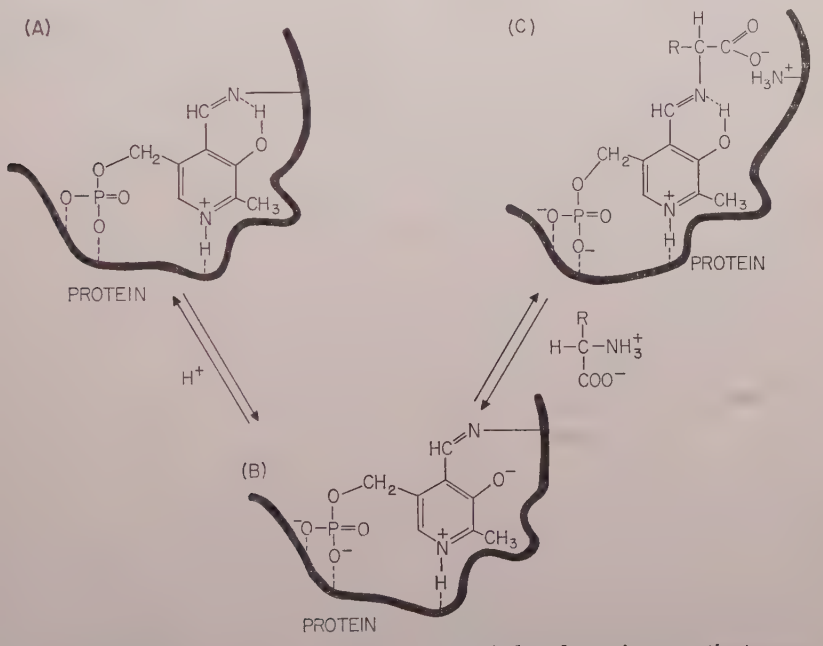


Fig. 12 Postulated nature of the pyridoxal form of the glutamic–aspartic transaminase. A, Inactive acidic form; B, active form; C, enzyme–substrate complex.

availability for interaction with sub-ate is limited by the nature and config-ation of the protein moiety and occurs adily only with dicarboxylic acids such aspartate and glutamate. These sub-ates are bound to the protein, presum-y via their carboxyl groups and displace selected substrates, all remain to be determined; figure 12 presents a conception of this consistent with present knowledge of the reaction. Continued investigation of highly purified transaminases will permit further clarification of each of these problems.

OPEN DISCUSSION

COHEN[1]: In 1950 Dr. P. S. Cammarata in my laboratory partially purified unresolved glutamic–oxaloacetic transaminase (the name we prefer to use for this enzyme). This preparation revealed an absorption peak at 408 mμ (*p*H 5.8), but, because of the presence of a contaminating heme pigment, no significance was attached to the observation. However, a sample was given to Dr. Snell for pyridoxal analysis and from the values reported we did not consider the amount of pyridoxal present sufficient to account for the absorption observed. The enzyme preparation has now been further purified and is free of the heme pigment; however, absorption studies have not been carried out with it. I assume that Drs. I. W. Sizer and W. T. Jenkins have been able to account for the absorption in terms of the pyridoxal (or pyridoxamine) content.

SNELL: Yes, they have. By heat or acid denaturation they can remove the protein and isolate the pyridoxal phosphate. The value of 2 moles of pyridoxal phosphate per mole of protein was obtained in this way. In a similar fashion, pyridoxamine phosphate is obtained from the pyridoxamine enzyme.

With reference to preferred nomenclature for glutamic–aspartic or glutamic–oxaloacetic transaminase, perhaps we should now insist that the enzyme be called glutamate–ketoglutarate transaminase, since these are the preferred substrates!

SHAPIRA[2]: Dr. Snell, since you mentioned that there are two pyridoxals or two pyridoxamines on one enzyme, have you considered the possibility that these could be located in close proximity? Possibly the anomalous *pK* is actually the *pK* resulting from the hydrogen that would be present when the pyridoxamine was linked to the pyridoxal. So, in effect, the actual enzyme contains one coenzyme in the form of the pyridoxal and one in the form of pyridoxamine.

SNELL: The extinction coefficient of the enzyme is too high in the pyridoxal form for this to occur. In the pyridoxamine form, of course, there is no pyridoxal left at all; so in the pyridoxamine form both are definitely in the form of pyridoxam phosphate. Also Jenkins and Sizer isol only pyridoxal from the enzyme isol in the presence of ketoglutarate; hence of the pyridoxal must be present as p doxal phosphate. It was suggested sev years ago by J. Baddiley that a sim interaction of pyridoxamine, a metal and pyridoxal might occur and be invol in nonenzymic and enzymic transam tion. Data on the pyridoxal and pyri amine forms of the transaminase wo seem to exclude this mechanism un these forms represent unphysiological tremes that do not occur normally du transamination. There is no evidence this is the case.

HANDLER[3]: Dr. George W. Schwert unable to attend this meeting but som the recent work in his laboratory b closely on the mechanisms described Dr. Snell. He and Dr. R. Shukuya obtai crystalline glutamic decarboxylase f *Escherichia coli*. In relatively acid s tions this enzyme shows an absorp maximum at 420 mμ similar to tha other pyridoxal phosphate enzymes. A tion of glutamic acid causes a small t sient depression in this absorption. S trofluorometry proved to be a much n useful technique for the study of this nomenon. Activation of only very sm amounts of the enzyme with light at mμ gave rise to pronounced fluoresce at 490 mμ. Addition of substrate resu in an instantaneous quenching of this e sion. The time period of quenching pends on the amount of glutamate ad Thus addition of 3 μmoles of subst to about 0.2 mg of enzyme resulted instantaneous quenching, which lasted about 20 seconds, after which the fluo cent intensity was regained. This nomenon strongly suggests that quenching arises from saturation of enzyme with substrate and that reco of intensity corresponds to exhaustion the substrate. At relatively alkaline however, the absorption maximum at mμ disappears and instead there is an sorption maximum at about 330 mμ, wh

[1] P. P. Cohen, University of Wisconsin.
[2] Raymond Shapira, Emory University.
[3] Philip Handler, Duke University.

annot be affected by the addition of substrate. These data are entirely compatible vith the Jenkins-Sizer mechanism, which)r. Snell described for a transaminase, hus supporting a unitarian concept of the nechanisms by which pyridoxal phosphate-dependent enzymes exert their catatic activity.

PIGMAN[4]: I wonder if the mechanism night not be improved slightly if you had ome positive mechanism for taking out he proton. This might help to explain ome of the stereospecificity of this reaction.

SNELL: Yes, that is so. I emphasized, ut perhaps not sufficiently, that the only atalytic action we have so far studied in nonenzymic systems is that of pyridoxal vith various metal salts. The rates here re very much less than those obtained with he enzyme; so that obviously subsidiary ctive sites in the enzyme are contributing o the over-all catalytic mechanism. Appropriately placed acidic or basic groups n the protein that would facilitate loss or ain of protons as required during the lectromeric shifts should greatly enhance ctivity of the nonenzymic system. We believe such subsidiary sites on the enzyme nust contribute to its catalytic effectiveness. If the unitary mechanism for pyridoxal-catalyzed reactions that we have presented is at all correct, then pyridoxal hosphate is the active site of each of the nzymes that contain it. Yet this isolated ctive site promotes these reactions at nuch lower rates than the enzyme itself. So I doubt if those who wish to isolate ctive sites of enzymes in general are ever oing to get single, small molecules that vill show the catalytic activity of the ntact enzyme. It will probably be necesary to isolate a segment of the protein hain that contains a variety of subsidiary ctive sites along with the primary active ite.

BUCHANAN[5]: Has there been any further dea during the last year about the funcion of maleic acid in the stabilization of he enzyme?

SNELL: No, so far as I know there has ot, but the spectral shifts with maleic cid are similar to those obtained initially vith ketoglutarate—a displacement of the bsorption maximum toward the longer wave length. This suggests that the maleate actually combines with and stabilizes two of the sites necessary for enzyme—substrate complex formation and that these are stabilized in a particular configuration so that heat denaturation will not separate them. We can visualize how the enzyme would be more resistant to denaturation under these circumstances, but the picture is still qualitative and perhaps not very helpful.

VESTLING[6]: I should like to make just one brief remark with respect to Dr. Buchanan's question. We have been trying to get rid of the glutamic–oxaloacetic transaminase in purifying maleic dehydrogenase from rat liver. We have the enzyme very highly purified but not yet crystalline. Our principal final job has been to get rid of transaminase. Early in the procedure we adopted a so-called heat-sensitization step in the presence of a variety of dicarboxylic acid anions, including maleate. There are very marked so-called protective effects, which we have simply regarded as the "gluing" together of a bit of structure by a double-barreled ionic attraction. I would prefer to think that this is a nice protective thing, and I would not worry too much at this stage about other implications of the effect of dicarboxylic acid anions.

HUGHES[7]: I would like to ask again about the optically active product from the nonenzymic reaction. Just how does that take place? I do not understand it. I think you said that an optically specific reaction occurs in the nonenzymic reaction.

SNELL: This occurs only when optically active reactants are used. That is, if we use L-phenylalanine and, in the presence of pyridoxal metal ions and pyruvate, we get phenylpyruvate together with an excess of L-alanine. The metal chelate initially formed contains coordinating positions that must be occupied by some of the excess L-phenylalanine, thus forming an optically active intermediate even though, when the structure shifts to the pyridox-

[4] Ward Pigman, University of Alabama Medical-Dental Schools.

[5] J. M. Buchanan, Massachusetts Institute of Technology.

[6] C. S. Vestling, University of Illinois.

[7] W. L. Hughes, Brookhaven National Laboratory.

amine–keto acid chelate, the asymmetry of the reactant molecule is destroyed. Essentially we have an L reactant in the transition state, with two choices as to whether it goes to an LL or LD product. Obviously the chances for forming each are not equal, since they are diastereomers. The LL configuration of product seems to be preferred in the two cases we have examined. Happily, this specificity turns out to be the same as that of the enzyme, where, with an L substrate we get an L product, and with a D substrate, a D product.

TODD[8]: I would make just one point in connection with this. I like the explanation that Dr. Snell has given, but I noticed that on the slide he showed us that he had specific rotations of ±1.5°. I would just like to know how he determines this because, on the concentrations given, a rough calculation seems to me to indicate that the observed rotation in 10 cm. would be about 0.01–0.02°. Was this observed photoelectrically or visually?

SNELL: This was done visually, but on amino acids isolated by column chromatography and examined at higher concentrations than those present in the reaction mixtures. Observed rotations were in the neighborhood of 0.04–0.08°; with ten consecutive readings, the average deviation was less than a tenth of this.

TODD: I just wondered what kind of polarimeter you used, since most visual instruments have a considerably lower accuracy.

SNELL: The instrument used was a Rudolph Model 80 with a 1-dm tube. We were very careful to repeat these observations several times before publication. We originally obtained the result with L-glutamate transaminating to L-alanine, but since both reactant and product had a plus rotation, we realized nobody would believe it. We hoped by obtaining a change in direction of rotation, as we did in the L-glutamate to L-phenylalanine experiment, to convince even the skeptics.

BRUICE[9]: Were the rate constants for your model systems calculated on the basis of the concentration of the metal Schiff base complex or on the total concentration of pyridoxal, metal, or amino acid? If the estimated rates were based on the latter, then the comparison to an enzymic system would be unfair. This is so, since at V_m would be following the breakdown of metal Schiff base–amino acid complex the enzyme.

SNELL: We have never calculated tual rate constants for the nonenzymic action. What comparisons have been ma were based on total pyridoxal concent tions, which as you point out, penalizes nonenzymic system unfairly since ma of the chelate complexes may be catal cally inactive.

[8] Alexander Todd, University Chemical Lab atory, Cambridge, England.

[9] T. C. Bruice, The Johns Hopkins School Medicine.

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he Hydrolysis of Peptide and Ester Bonds y Proteolytic Enzymes

HANS NEURATH AND BRIAN S. HARTLEY[1]
Department of Biochemistry, University of Washington, Seattle

Application of classical enzyme kinetics the reaction of proteolytic enzymes with -acyl amino acid derivatives, partic- arly peptides, amides, and esters, has ovided a foundation for the delineation enzyme specificity and the elucidation the reaction mechanism. A simple for- ulation in the sense of Michaelis and enten, as modified by Briggs and Hal- ne, seemed adequate for an interpreta- on of the kinetic data. This formulation dmittedly is subject to refinement, but a ber review of the more recent literature ises some doubt about how much further arification can be achieved by processes kinetic sophistication. Indeed, the sim- e formulation involving K_m, the Michaelis nstant, and k_3, the rate constant for the composition of the Michaelis complex, nnot be adequately described in terms of own chemical mechanisms. Even in the rely descriptive sense of defining the ecificity of these enzymes, these studies ve their limitations, since no reliable ediction can yet be made of the way in hich these enzymes will attack a large ptide or protein—their physiological sub- rates.

Studies of the reaction of chymotrypsin d trypsin with "nonspecific" substrates d inhibitors that act as acylating or osphorylating agents have opened new proaches to the study of the chemical ature of the intermediates in the hydroly- s of more-typical substrates and have ex- nded the kinetic formulation to include ecific chemical reactions in the forma- on and decomposition of intermediates. lthough this combination of kinetic and emical evidence thus seems to have ought us closer to the mechanistic de- ription of enzymic catalysis by these pro- ases, the nature of the groups on the en- me molecule that participate in the reac- tion and their spatial relation within the protein structure need to be elucidated if the entire process is to be described in terms of structural chemistry. Advances in enzyme kinetics are thus clearly coupled to progress in protein chemistry. It is our purpose in this paper to review the current situation in this field and to show the interrelation of kinetic, chemical, and structural considerations in the analysis of the reaction of a typical proteolytic enzyme, namely, chymotrypsin, with its substrates and inhibitors.

THE CONTRIBUTION OF CLASSICAL ENZYME KINETICS

Specific substrates and inhibitors

It is pleasant to be able to report wide agreement that the reaction of trypsin and chymotrypsin (ChTr) with a large range of specific substrates and competitive inhibitors can be expressed in terms of classical enzyme kinetics [eq. (1), fig. 1].

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P\,; \quad K_m = \frac{k_2 + k_3}{k_1}$$

$$E + I \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} EI\,; \quad K_i = \frac{k'_2}{k'_1} \qquad (1)$$

Figure 1

In some cases, the reaction products act as competitive inhibitors, but this may be allowed for in calculating K_m and k_3 (Huang and Niemann, '51). Our argument will be restricted to studies with α-ChTr, since an impressive amount of information is available, largely owing to the

[1] Permanent address: Department of Biochemistry, University of Cambridge, Cambridge, England. Presently Helen Hay Whitney Fellow at the University of Washington.

careful work of Niemann and his collaborators. Adequate parallels exist, however, for the enzyme trypsin.

The specificity of α-ChTr has been mapped out by use of a series of structural analogs of peptide substrates (Neurath and Schwert, '50). Early conclusions that tyrosine, phenylalanine, and tryptophan residues (Bergmann and Fruton, '41; Kaufman and Neurath, '49) are optimum sites of attack in synthetic substrates have stood the test of much investigation. Thus, in the series of esters shown in table 1, the aromatic amino acid derivatives show both greater affinity for the enzyme and greater lability. However, the π-electrons of the aromatic ring do not appear to be essential for the activity, since *N*-acetyl-L-hexahydrophenylalaninamide has almost the same kinetic constants as *N*-acetyl-L-phenylalaninamide (Jennings and Niemann, '53).

K_m as a measure of "affinity." A simp fying conclusion, which can now be dra about the hydrolysis of almost all su substrates by ChTr, is that K_m appro mates to K_s—the true dissociation consta of the enzyme—substrate complex, whi is therefore an inverse measure of t affinity of the substrate for the active c ter. Evidence comes from a number sources.

Table 2 shows kinetic constants for series of *N*-acetyl-L-tyrosine substrates which the labile group (—X) is vari In the first four of these, k_3 varies ove fiftyfold range, whereas K_m remains rou ly constant and of the same order as t inhibition constant (K_i) for the corr ponding D enantiomorph. Hence the ra $(k_2 + k_3)/k_1$ is independent of k_3, and m approximate to $k_2/k_1 = K_s$. We can apply this argument to *N*-acetyl-L-tyrosi ethyl ester (ATEE), but *p*H studies to

TABLE 1

Hydrolysis of N-acetyl amino acid esters by α-chymotrypsin[a]

Substrate	K_m	k_3[b]	Reference
	$M \times 10^3$	sec^{-1}	
Ac-L-Try-OEt[c]	0.09	50.6	Cunningham and Brown, '56
Ac-L-Tyr-OEt[c]	0.7	193	Cunningham and Brown, '56
Ac-L-Phe-OEt	1.1	173	Hammond and Gutfreund, '55
Ac-L-Leu-OMe	—	>4	Applewhite, Waite, and Niemann, '58
Ac-L-Ileu-OMe	—	~ 0.07	Applewhite, Waite, and Niemann, '58
Ac-L-Val-OMe	108	0.15	Martin and Niemann, '58
Ac-Gly-OMe	10	0.008	Wolf and Niemann, '59

[a] *p*H 7.9, 25°C.
[b] Assuming mol. wt. 25,000 and 16.0% *N* for α-ChTr.
[c] In the presence of 0.1 *M* $CaCl_2$.

TABLE 2

Hydrolysis of N-acetyl-L-tyrosine substrates by α-chymotrypsin, and competitive inhibition by D-isomers[a]

Acetyl tyrosine—X —X	L substrate		D inhibitor	Reference
	K_m	k_3	K_i	
	$M \times 10^3$	sec^{-1}	$M \times 10^3$	
—$NHNH_2$	22	0.05	7.5	Lutwack *et al.*, '57; Foster and Niemann, '55,a,b
—NH_2	32	0.17	12	Manning and Niemann, '58
—$NHCH_2CONH_2$	23	0.50	—	Foster and Niemann, '55a,b
—NHOH	43	2.20	7.5	Foster and Niemann, '55a,b Foster *et al.*, '55
—OC_2H_5	0.7[b]	193[b]	5.0	Cunningham and Brown, '56; Foster and Niemann, '55a,b

[a] *p*H 7.9, 25°C.
[b] In the presence of 0.1 *M* $CaCl_2$.

iscussed later show that here, too, $K_m =$
s.

A similar situation is observed when the cyl group is varied in a series of *N*-acyl-L-rosinamides (table 3) (Manning and iemann, '58). Values of K_m for the L sub-rates are of the same order as K_i for the competitive inhibitors (which must be inding constants), and a tenfold variation k_3 is not reflected in K_m. Hence, here too, appears probable that $K_m = K_s$.

For individual substrates, the conclusion at $K_m = K_s$ was reached by investigating ie effects of buffer (Kerr and Niemann, 58), salts (Martin and Niemann, '58), ucrose (Shine and Niemann, '56), or or-anic solvents (Applewhite, Martin, and iemann, '58) on the kinetic constants. hus representative data in table 4 for

TABLE 3

Hydrolysis of N-acyl-L-tyrosinamides by α-chymotrypsin and competitive inhibition by D isomers. pH 7.9, 25°C.

(Manning and Niemann, '58)

RCO-tyrosinamide acyl residue: (RCO—)	L substrate		D inhibitor
	K_m	k_3	K_i
	$M \times 10^3$	sec^{-1}	$M \times 10^3$
HCO—	12	0.03	—
C_2H_5OCO—	6.4	0.05	21
CH_3CO—	32	0.17	12
CF_3CO—	26	0.17	20
CH_2ClCO—	27	0.27	6.5
 —CO—	2.5	0.27	—
 —CO—	12	0.33	9

TABLE 4

ffect of organic solvents on the hydrolysis of ethyl hippurate by chymotrypsin. pH 7.9, 25°C.

(Applewhite, Martin, and Niemann, '58)

Solvent	Volume %	K_m	k_3
		$M \times 10^3$	sec^{-1}
one added	—	7.55	0.19
cetone	5	12	0.19
	10	21	0.19
	15	28	0.18
	20	40	0.18
ioxane	0.4	9.1	0.19
	5	25	0.19
	10	50	0.19
	15	92	0.18

the chymotryptic hydrolysis of methyl hippurate (Applewhite, Martin, and Niemann, '58) show that organic solvents increase the value of K_m, whereas k_3 remains constant. In this context, the effect of alcohols should be interpreted with caution because of their probable reaction with the acyl—enzyme (Balls and Wood, '56). An opposite effect, i.e., k_3 increases while K_m remains constant, is found in the chymotryptic hydrolysis of α-*N*-nicotinyl-L-tyrosine hydrazide, in the presence of increasing concentrations of Tris [tris(hydroxymethyl)aminomethane] buffer (Kerr and Niemann, '58). In all these cases, the conclusion that K_m is an equilibrium constant seems inescapable.

Effect of pH. In detailed studies of the kinetics of α-ChTr-catalyzed hydrolysis of specific substrates, the conclusion may again be drawn that K_m reflects the equilibrium constant K_s. Thus Hammond and Gutfreund ('55) showed that, for the chymotryptic hydrolysis of acetyl-L-phenylalanine ethyl ester, K_m is practically constant over the range *p*H 6.5–8.0, whereas the value of k_3 seems to be governed by the ionization of a group in the enzyme of $pK_a = 6.85$ at 25°C., the un-ionized species being required for activity. Similar conclusions for the substrates ATEE and α-*N*-acetyl-L-tryptophan ethyl ester, were reached by Cunningham and Brown ('56). They found that k_3 was dependent on the ionization of a group in the enzyme of $pK_a = 6.7$ at 25°C., and ΔH of ionization = 11 kcal/mole, whereas K_m was practically constant between *p*H 6.0 and 8.0. With *N*-acetyl-L-tyrosinamide, Gutfreund and Sturtevant ('56a) ascribe $pK_a = 6.7$ at 25°C. to the group in the enzyme that controls the value of k_3.

This ionizing group has plausibly been ascribed to the imidazole ring of a histidine residue, but whatever its origin we may conclude that it controls k_3 but not K_m.

The reaction of chymotrypsin with p-nitrophenyl acetate

Hartley and Kilby ('52, '54), as part of an investigation of the inhibition of ChTr by organophosphates, discovered that *p*-nitrophenyl acetate (NPA) could function as a substrate for ChTr. The reaction was characterized kinetically by an initi-

ally rapid liberation of *p*-nitrophenol, followed by a linear reaction phase in the steady state. The initial "burst" was ascribed to the acylation of the enzyme and the steady-state phase to the continuous hydrolytic deacylation and reacylation. The two steps can be separated from each other, since, below *p*H 5.5, no turnover occurs (Balls and Aldrich, '55; Balls and Wood, '56). In this connection, it may be helpful to point out that NPA is not a typical ester, but resembles, in many of its properties, an acid anhydride (Hartley and Kilby, '54).

A simple formulation, omitting ionization steps (which can always be corrected for by extrapolation to zero hydrogen ion concentration), is given in equation (2) (Gutfreund and Sturtevant, '56a) [eq. (2), fig. 2], where ER is the acyl intermediate,

$$E + RX \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}RX \xrightarrow{k_3} ER \xrightarrow{k_5} E + R \qquad (2)$$
$$+$$
$$X$$

Figure 2

as measured by the initial "burst," whereas deacylation of the enzyme is measured by the rate-controlling, slow linear stage of the reaction. The question of the formation of a Michaelis-Menten type of intermediate during acylation (E·RX) has been a matter of debate (Gutfreund and Sturtevant, '56b; Dixon and Neurath, '57a), but, as will be shown, all available data are compatible with the inclusion of this intermediate in the formulation of the reaction process.

Experimentally measurable kinet quantities include the following: the r constants for the turnover phase (k_5) a for the initial liberation of *p*-nitrophe (k_3); the dependence of both of these substrate concentration, K_m (acylatio and K_m (turnover). These parameters w now be considered in relation to the re tion scheme represented by equation (

Turnover rates. A typical progress cur of the reaction of ChTr with NPA at *p*H is shown in figure 3, curve 1; curve 2 rep

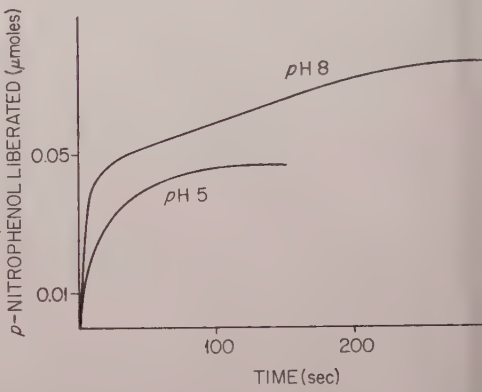

Fig. 3 Progress curves of the reaction ChTr with NPA.

sents for comparison the reaction at *p*H where the deacylation reaction does occur. The linear portion of curve 1 rep sents the turnover phase, and although experimental conditions used by vari investigators are not strictly comparab the reported values for k_5, the veloc constant, at infinite substrate concent tion, vary within relatively narrow lim as shown in table 5. For experimen

TABLE 5

Experimental values of k_5 for p-nitrophenyl acetate

k_5[a]	Protocol	References
$sec^{-1} \times 10^3$		
25.3	25°C., *p*H 7.6, 5% isopropanol, 0.1 *M* phosphate	Hartley and Kilby, '54
25.4	27°C., *p*H 6.45–7.75, 20% isopropanol, 0.05 *M* phosphate	Gutfreund and Sturtevant, '56a
12.1	28°C., *p*H 6.20–7.60, 2% acetone, 0.07 *M* phosphate	McDonald and Balls, '57
11.3	25°C., *p*H 7–8, 0.4% dioxane, 0.005 *M* Tris-maleate, 0.5 *M* KCl	Awad and Neurath (unpublished)

[a] Extrapolated to $(H^+) = 0$, assuming $pK = 7.3$.

reasons, to be documented in another publication (Awad and Neurath, unpublished), we shall adopt the value of $k_5 = 12 \times 10^{-3}$ sec^{-1} as the most probable one. Various lines of reasoning, presented elsewhere in this discussion, lead to the conclusion that k_5 is a measure of the rate of deacylation of ER. This view is strongly supported by the satisfactory agreement between k_5 and the rates of deacylation measured by other, indirect methods. The latter include (1) the rate of appearance of enzymic activity toward the synthetic substrate, ATEE, when monoacetyl-δ-ChTr, prepared according to the method of Balls and Aldrich ('55), is allowed to become spontaneously deacylated at *p*H 7.0 (Dixon and Neurath, '57a); and (2) the rate of disappearance of the absorption peak at 245 mμ when monoacetyl-δ-ChTr is allowed to become deacylated at *p*H 9 (Dixon and Neurath, '57c).

The rate of reappearance of enzymic activity toward ATEE during the deacylation of monoacetyl-δ-ChTr corresponded to a first-order rate constant (with respect to time) of 7.0×10^{-3} sec^{-1} at 10°C. extrapolated to zero hydrogen ion concentration. When the rate of decay of the absorption at 245 mμ, occurring during the deacylation at *p*H 9, 10°C., is expressed as a first-order constant, a value 2.1×10^{-3} sec^{-1} is obtained. In view of the approximations involved in these measurements and in their interpretation, the agreement between the three sets of data may be considered reasonably satisfactory.

The dependence of turnover rate on initial substrate concentration permits the calculation of K_m (turnover). This constant has been determined by Awad and Neurath (unpublished) and by Spencer and Sturtevant ('59) and, although the experimental conditions were not exactly the same, the values obtained are in good agreement, i.e., K_m (turnover) = 40–50 $\times$ 10^{-6} *M* (25°C., *p*H 8, ionic strength 0.1).

Acylation. The initial rate of liberation of *p*-nitrophenol may be as much as 300 times as fast as the turnover rate and hence requires special conditions. Gutfreund and Sturtevant ('56a, b) used the stopped-flow technique to measure fast reactions, whereas Dixon and Neurath ('57a) and Awad and Neurath (unpublished) resorted to measurements at lower *p*H and lower temperature to retard the acylation reaction. Gutfreund and Sturtevant ('56a, b) calculated first-order rate constants as a function of initial substrate concentration, and since these could be fitted to Michaelis-Menten kinetics, they concluded that the step characterized by k_3 [see eq. (2)] was being measured and that a Michaelis-Menten type intermediate existed in this phase of the reaction. Dixon and Neurath ('57a), working at a lower *p*H (5.5) and at lower substrate:enzyme ratios (0.4–8, as compared to ~5–100 used by Gutfreund and Sturtevant), interpreted their data as second-order constants and hence questioned the existence of a Michaelis-Menten intermediate. However, when Dixon and Neurath's data are converted to first-order constants and fitted to Michaelis-Menten kinetics, satisfactory agreement is obtained between these two sets of data as well as with our more-recent data (Awad and Neurath, unpublished), as shown in table 6. The value of $k_3 = 4$ sec^{-1} may therefore be accepted with considerable confidence, a value that

TABLE 6

Experimental values of rates of acylation (k_3)

k_3[a]	Protocol	References
sec^{-1}		
3.8	25°C., *p*H 7.75, 20% isopropanol, 0.05 *M* phosphate, S:E = 5.5–110	Gutfreund and Sturtevant, '56a
4.6	10°C., *p*H 5.5, 0.2 *M* citrate S:E = 0.4–8	Dixon and Neurath, '57a
3.9	12°C., *p*H 7.9, 0.1 *M* KCl, 0.01 *M* Tris, S:E = 6.3	Awad and Neurath (unpublished)

[a] Extrapolated to (S) = ∞ and (H^+) = 0, assuming $pK = 6.9$.

is considerably lower than $k_3 = 193$ sec^{-1} for the chymotryptic hydrolysis of ATEE (see table 2).

K_m values of the acylation reaction have been reported by Gutfreund and Sturtevant ('56a, b) and, although the measurements were carried out in the presence of an organic solvent (20% isopropanol), the value of K_m (acylation)$= 5 \times 10^{-3}$ M can be accepted as probable.

Significance of kinetic constants. On the basis of the reaction mechanism represented by equation (2), the experimental parameters, summarized in table 7, may be

TABLE 7

Kinetic constants of the reaction of chymotrypsin with p-nitrophenyl acetate

Constant	
k_3	4 sec^{-1}
k_5	12×10^{-3} sec^{-1}
K_m (acylation)	5×10^{-3} M
K_m (deacylation)	40×10^{-6} M

related to the various rate constants appearing in the equation. As previously mentioned, the specific rate constant for the turnover reaction may be identified with k_5, which is evidently the slowest and hence the rate-determining step. The rate constant characterizing the initial "burst" reaction represents k_3. From Dixon and Webb ('58) for the case of equation (2), assuming $k_5 << k_3$, which is experimentally justified, it follows that K_m (acylation)$= (k_3/k_5)K_m$ (deacylation). From this, K_m (acylation)$= 10 \times 10^{-3}$ M, a value in good agreement with the observed K_m (acylation)$= 5 \times 10^{-3}$ M (values at *p*H 8, 25°C.).

Dependence on pH and temperature. The dependence of both the acylation and deacylation reactions on *p*H can be expressed as being influenced by the ionization of a single group having a *pK* in the neighborhood of neutrality. Although the measurements carried out by different investigators were not under strictly comparable experimental conditions, the agreement is surprisingly good (table 8). The following conclusions may be drawn from these data: (1) The *pK* of the ionizing group involved in acetylation is somewhat lower than that of the group involved in deacetylation; (2) the *pK* of the group involved in acetylation by NPA is approximately the same as that for the hydrolysi of specific substrates; (3) the *p*H depend ence of the reactions catalyzed by trypsi is similar to that involved in chymotrypti catalysis and presumably involves a sim lar group (table 8); and (4) the magn tude of the *pK* values is strongly suggestiv of the ionization of the imidazolyl group c a histidine side chain. In passing, it shoul be noted that the *p*H dependence of ChT and the reaction of various other esterase with organophosphorus compounds ca be similarly ascribed to the ionization of group of the same type (table 8).

Limited data are available on the tem perature dependence of the reaction c ChTr with NPA or with *N*-acyl amin acid esters. From these, conclusions ca be drawn about the enthalpy of the ioni ing group (pK_a) that controls the rate-lim iting step (Cunningham and Brown, '56) The reported values are consistent wit that to be expected for an imidazolyl group seem to exclude a carboxyl group, but can not in themselves exclude an α- or ε-amin group.

Acyl—enzyme formation by "specific" substrates

We cannot decide whether the reactio sequence for NPA [eq. (2)] applies equall to the hydrolysis of "specific" substrates b ChTr simply by examining the kineti constants for the latter. Thus table shows that, for a series of substrates of th form *N*-acetyl-L-tyrosine—X, the rate-limi ing step (k_3 in fig. 1) varies from 0.0 sec^{-1} for the hydrazide to 193 sec^{-1} fo the ethyl ester. If deacylation of the en zyme (k_5 in fig. 2) were the rate-limitin step, all these substrates should show th same maximum velocity, since the sam acyl—enzyme would be formed from each Hence k_5, if it exists, must be much large than k_3, and the rate-limiting step in th hydrolysis would be the formation of th acyl—enzyme.

This argument cannot be applied to th most rapid reaction in table 2, the hydroly sis of ATEE, where the rate-limiting ste may be either k_3 or k_5. However, an inte esting comparison may be made betwee the kinetic constants for chymotryptic hy drolysis of ATEE, NPA, and a new sub strate, studied by Martin *et al.* ('59), N

TABLE 8

pH dependence

pK	System	Protocol	References
Turnover			
7.28	NPA + α-ChTr	27°C., 20% isopropanol, 0.05 *M* PO_4	Gutfreund and Sturtevant, '56b
7.32	NPA + δ-ChTr	25°C., 0.4% dioxane, 0.005 *M* Tris, 0.005 *M* maleate, 0.05 *M* KCl	Awad and Neurath (unpublished)
7.44	DNPA + δ-ChTr	As above	Awad and Neurath (unpublished)
7.2	NPA + α-ChTr	28°C., 2% acetone, 0.07 *M* PO_4	McDonald and Balls, '57
6.96	Hydrolysis of acetyl-δ-ChTr	25°C., ATEE assay mixture	Dixon and Neurath, '57a
"Acylation"			
6.22	NPA + δ-ChTr	3°C., 0.2 *M* citrate	Dixon and Neurath, '57a
6.7	DNPA + α-ChTr	25°C., 20% isopropanol, 0.05 *M* PO_4, 0.1 *M* NaCl	Gutfreund and Sturtevant, '56a
7.0	NPA + δ-ChTr	5°C., 0.4% dioxane, 0.01 *M* Tris, 0.01 *M* maleate, 0.1 *M* KCl	Awad and Neurath (unpublished)
6.6	E600 + α-ChTr	25°C., 5% isopropanol, 0.1 *M* phosphate	Hartley, '56
Specific substrates			
6.82	ATEE + δ-ChTr	25°C., ATEE assay mixture	Dixon and Neurath, '57a
6.7	ATA + α-ChTr	25°C., 20% isopropanol, 0.025 *M* maleate, 0.1 *M* NaCl	Gutfreund and Sturtevant, '56a
6.85	APhEE + α-ChTr	25°C., 0.05 *M* PO_4, 0.1 *M* NaCl	Hammond and Gutfreund, '55
Other esterases			
7.00	NPA + trypsin	25°C., phosphate	Dixon and Neurath, '57b
6.02	NPA + trypsin	25°C., acetate	Dixon and Neurath, '57b
6.26	NPA + trypsin	25°C., cacodylate	Dixon and Neurath, '57b
6.25	BAEE + trypsin	25°C., 0.01 *M* phosphate, 0.1 *M* NaCl	Gutfreund, '55
6.08	BAEE + trypsin	35°C., 0.01 *M* phosphate, 0.1 *M* NaCl	Gutfreund, '55
6.6–7.1	DFP + various esterases		Mounter *et al.*, '57

DNPA = 2,4-dinitrophenyl acetate.
E600 = *p*-nitrophenyldiethyl phosphate.
ATA = *N*-acetyl-L-tyrosinamide.
APhEE = *N*-acetyl-L-phenylalaninamide.

TABLE 9

Kinetic constants for chymotrypsin substrates, pH 8.0

Substrate	K_m	k_3	Reference
	$M \times 10^3$	sec^{-1}	
Acetyl-L-Tyr-ethyl ester[a]	0.7	193	Cunningham and Brown, '56
Carbobenzoxy-L-Tyr-nitrophenyl ester[b]	0.03	553	Martin *et al.*, '59
Acetyl-*p*-nitrophenyl ester (NPA):[c]			
Acylation	5.0	4	See table 7
Turnover	0.04	0.012	See table 7

[a] 25°C., 0.1 *M* $CaCl_2$.
[b] 30°C., 0.1 *M* $CaCl_2$, 12% MeOH.
[c] 25°C., 0.5 *M* KCl, 0.4% dioxane.

carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester. Table 9 shows that K_m and k_3 for the latter are of the same order as those for ATEE, but that both tyrosine esters are hydrolyzed at least 16,000 times as rapidly as NPA. It follows, therefore, that regardless of whether acylation or deacylation is rate limiting for the hydrolysis of the tyrosine esters, the acyltyrosyl—enzyme must be deacylated at a rate at least 16,000 times as fast as the equivalent acetyl—enzyme. Humility compels us to emphasize that no mechanism of action has been offered that would satisfactorily explain this—perhaps the most important characteristic of the enzyme.

Returning, however, to more mundane fields, it would be interesting to know whether an initial "burst" of nitrophenol occurs during the hydrolysis of such acyl—tyrosine nitrophenyl esters as Martin *et al.* ('59) have used. This might enable us to decide, as with NPA, whether hydrolysis of the acyl—enzyme is rate limiting. (Note added in proof: Dr. H. Gutfreund recently informed us that he has investigated the reaction of *N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester with chymotrypsin by the use of the stopped-flow technique and was able to record the initial "burst.") In trypsin, this conclusion can legitimately be drawn. Schwert and Eisenberg ('49) have shown that in the tryptic hydrolysis of a series of α-*N*-benzoyl-L-arginine esters, k_3 remains constant whereas the alcohol residue varies from methyl to benzyl or cyclohexyl, suggesting that deacylation of the benzoylarginyl—enzyme is the step being measured.

Although, with ChTr, the existence of an acyl—enzyme cannot be so directly inferred from the kinetics, there is ample circumstantial evidence for its existenc Thus transpeptidation between substrat such as α-*N*-benzoyl-L-tyrosinamide an -glycinamide (Fruton *et al.*, '51) or tran esterification of *N*-benzoyl-L-phenylalanin ethyl ester plus methanol to give th methyl ester (Bender and Kemp, '57b), a best explained on the hypothesis of a acyl—enzyme.

Studies of O^{18} exchange catalyzed b ChTr, which were first demonstrated b Sprinson and Rittenberg ('51), point t the existence of an acyl—enzyme. Th reaction occurs with a "virtual substrat such as carbobenzoxy-L-phenylalanine, N acetyldibromo-L-tyrosine (Doherty an Vaslow, '52), benzoyl-L-phenylalanine, c acetyl-L-tryptophan (Bender and Kemp '57a), but not with their D enantiomorph It may be expressed by Michaelis-Mente kinetics (Vaslow, '56), and K_m is identic with the equilibrium constant determine by dialysis equilibrium (Doherty and Va low, '52) or with K_i when the compoun acts as competitive inhibitor of anothe substrate (Bender and Kemp, '57a). Ben der and Kemp ('57b) have shown tha during hydrolysis by ChTr of O^{18}-labele ethyl - β - phenylpropionate or benzoyl - L phenylalanine ethyl ester, fission at th

—bond occurs without O^{18} e change, whereas alkaline hydrolysis is a companied by O^{18} exchange. They explai this by postulating an acyl—enzyme inte mediate. In the absence of further ev dence, we are therefore persuaded to a sume such an intermediate in all catalyse by ChTr.

The major conclusion that may be drawn from the kinetics of reaction of ChTr with specific substrates, with "virtual" substrates, and with NPA and other acylating reagents may be summarized as follows: The reaction involves an acylation of the enzyme through a Michaelis-Menten type of intermediate, followed by deacylation. In certain, but by no means all, cases, deacylation is the rate-controlling step but the rate of deacylation is tremendously dependent on the nature of the acyl substituents. The imidazolyl group of a histidine side chain probably controls both acylation and deacylation but has no effect on the formation of the Michaelis-Menten intermediate. These conclusions are also consistent with the reaction of ChTr and trypsin with diisopropylfluorophosphate (DFP) and related organophosphates where k_5, the rate constant of dephosphorylation, is practically zero, and k_1 is rate limiting (Hartley and Kilby, '52) (see fig. 2).

THE CHEMICAL NATURE OF THE ACYL—ENZYME

There is overwhelming evidence that a specific serine side chain is the ultimate site of acylation, or phosphorylation, in the reaction of ChTr, trypsin, and other esterases with acylating and phosphorylating agents under conditions leading to monosubstituted derivatives. The evidence has been considered in detail in a previous review (Dixon, Neurath, and Pechère, '58) and is based on direct proof obtained by isolation of acyl or phosphoryl peptides. The simultaneous, if not exclusive participation of a histidine side chain in these reactions is suggested by indirect evidence that includes, as previously mentioned, (1) the *p*H dependence of the reaction of these esterases with substrates, including NPA; (2) the transient appearance of a species having maximum absorption at 245 mμ (Dixon and Neurath, '57c); (3) the abolition of the reaction with DFP after photooxidation (Weil *et al.*, '53), which destroys one out of two histidine residues and some tryptophan; and (4) the inactivity of the enzyme after blocking one of the two histidine side chains by reaction with 1-fluoro-2,4-dinitrobenzene (Whitaker and Jandorf, '56).

Certain other groups can definitely be excluded from a direct involvement in the enzymic reaction of these esterases, including sulfhydryl groups since they do not exist in ChTr or trypsin (Green and Neurath, '54) or free amino groups. Thus Chervenka and Wilcox ('56b) showed that conversion of the 13 ε-amino groups of chymotrypsinogen into guanidino groups had no effect on activation of the zymogen or on the catalytic activity of the activated product. Reaction of the N-terminal alanine group in α-ChTr with 1-fluoro-2,4-dinitrobenzene did not destroy the activity, but the N-terminal isoleucine group was unreactive and cannot be categorically excluded (Massey and Hartley, '56). Acylation of the amino groups of ChTr (Jansen *et al.*, '51) or trypsin (Fraenkel-Conrat *et al.*, '49) is known to be without effect on activity, and reaction of the single N-terminal residue of chymotrypsinogen (cystine) with carbon disulfide does not hinder activation of the zymogen (Chervenka and Wilcox, '56a). No definitive studies have been reported to test similarly the possible participation of other reactive side chains of the enzyme, such as tryptophan, tyrosine, or carboxyl groups.

Structural relations

The structural relation of the serine and histidine side chains believed to be part of the catalytic site must be close, since one but not two acyl or phosphoryl groups can specifically react with one enzyme molecule and since diisopropylphosphoryl-ChTr is unreactive as an enzyme and cannot form a monoacetyl derivative (Hartley and Kilby, '54). Studies of the effects of denaturation on the formation of diisopropylphosphoryl-trypsin and of the reactivity of monoacetyl-δ-ChTr likewise point toward a close proximity in space between these two amino acid side chains (Dixon *et al.*, '56).

Formulation of the enzymic acylation and deacylation mechanism in terms of a specific configuration of two amino acid side chains, serine and histidine, was proposed by Cunningham ('57) and by Dixon and Neurath ('57c), in terms of a hydrogen-bonded structure of the type Ser.H.His, histidine being involved in the acylation and deacylation of serine. Although this

scheme, or some variation thereof, is mechanistically probably correct, it appears insufficient to explain enzymic catalysis. The facts that both acylation and deacylation of the enzyme are so strongly influenced by the very structural components of the substrate that determine its affinity for the enzyme and may cause a several thousandfold variation in rate, cannot be explained by the chemical characteristics of the acyl group alone. It is more likely that configurational changes in the enzyme, induced perhaps by configurational adaptation to the substrate, create a constellation that provides the energetic advantage characteristic of enzymic catalysis. This problem seems as yet beyond the reach of structural description and clearly focuses attention on the lack of understanding in an area that may well be the heart of enzymology.

Nevertheless, a solution to this problem must encompass the location within the enzyme structure of the serine and histidine side chains involved in acylation and deacylation, together with those other amino acid residues that contribute to the specificity of the enzyme. Since these unique serine and histidine side chains in trypsin are not adjacent to each other along an α-helix (Dixon, Kauffman, and Neurath, '58) and since the substrate specificities of ChTr and trypsin are so very different, a twin attack on the amino acid sequence in these two enzymes, now in progress in this laboratory, becomes particularly important. Recent advances in the elucidation of the primary structure of ChTr, about which considerably more is known at this time than about trypsin (Neurath *et al.*, '59) will not be considered here, but in the course of the discussion Dr. Hartley will review the current situation of the problem. We seem today to be on the threshold of an understanding of protein structure and properties which will surely dictate the future of enzymology.

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OPEN DISCUSSION

(Includes discussions that followed this paper and the one by B. S. Hartley and also discussions that took place during one o the evening sessions of the conference.)

HUGHES[2]: Are any of these fragment active enzymically?

HARTLEY: No. Unfortunately, when ever you oxidize, denature, or do anythin much to what we call the tertiary structur of this enzyme the activity vanishes, an an unavoidable conclusion is that the ele ments of the activity center are arrange rather critically on different parts of thi long chain.

BERNHARD[3]: I should like to commen on the question of the tertiary folding in terrelation between the substrate and th enzyme. I think perhaps we can get a littl too enthusiastic about the complicate structure of the protein and lose some o the simple models proposed earlier fo specificity. We studied a number of sub strates of ChTr of the type $R^1CONHC(R)X$ where C—X is the bond that is broken an R and R^1 are the chemical groups we ar varying. In the usual model substrates fo ChTr there is a large ring at R. R^1 can b something nonspecific like methyl. If w put the large ring at R^1 and the small grou at R, we find that this latter derivativ binds as well as the usual substrate. Th rate at which it is split, however, is abou 1/1000 the rate with the usual substrate Dr. Neurath might ascribe this to som very complicated sort of refolding of th enzyme, but we have a much simpler ex planation; namely, that the substrate goe in the wrong way. We can show that thi is true by removing both rings and sub stituting two small R groups. Such a sub strate can no longer bind tightly, but in highly concentrated solution we can ob serve Michaelis-Menten type kinetics. Th limiting rate of hydrolysis is similar to tha for the fast specific substrate. Perhap through evolution the enzyme has learne to deal with wrong substrates. One way deals with them is to have an alternat binding method built into the molecul We think that this may (at least for ChTr) be a method for dealing with th wrong substrates, and we should like

[2] W. L. Hughes, Brookhaven National Labor tory.

[3] S. A. Bernhard, National Institutes of Healt Bethesda.

think of the enzyme site as rather rigid, as far as its specificity goes.

HARTLEY: As I have explained, the whole digest (where presumably everything is present) is inactive; so it is unlikely that bits of it would be active if separated and recombined.

KOSHLAND[4]: Dr. Neurath, have you ever added imidazole to separated acyl peptides to see if the combination reacts especially rapidly?

NEURATH: We have never tried it.

P. P. COHEN[5]: We have tried synthetic tripeptides of the asparagine series, the glycine-asparagine series, and lysine and found that, in the presence of large concentrations of histidine and in the absence of ChTr, there was no reaction.

BENDER[6]: We measured the methanolysis of acetyl-L-phenylalanine methyl-C^{14} ester in methanol–water solutions. The methanolysis reaction is measured by determining the decrease in activity of the substrate as it is hydrolyzed. Some of Professor Neurath's kinetics involved measurements in alcohol–water solutions. Our data indicate that, with about 1–2% methanol, the methanolysis can compete with the hydrolysis successfully and thus methanol can affect the over-all kinetics.

Furthermore, a kinetic analysis indicates that there is a water or, alternatively, a methanol site on the enzyme. If we assume this, then the reaction of methanol with most acyl—enzymes is faster than the reaction of water with acyl—enzymes by a factor of something like 8.

HESS[7]: Dr. Neurath's summary seems to be consistent with the data presented on ChTr-catalyzed reactions. Dr. M. A. Marini and I have obtained evidence that two stable forms of monoacetyl—ChTr exist. The deacylation of only one of these intermediates is accompanied by a change in absorption at 245 mμ. It is this change that suggested that the deacylation of the enzyme proceeds via an *N*-acylimidazole derivative. Very clearly, the existence of two different acyl—enzymes has to be taken into consideration when interpreting the mechanism of ChTr-catalyzed reactions.

NEURATH: It seems to me entirely possible that more than one intermediate exists, but I also think that, until and unless we know more about the region on the protein usually referred to as the active site, it would be very difficult to insist on any specific transition state. Even with the relatively simple types of organic reaction that we have heard about in this meeting, we are hard pressed to define the configuration of transient intermediates, and I think the problem here is magnified manyfold.

STURTEVANT[8]: One of the crucial points about the present status of the ChTr problem is whether the so-called specific enzymes follow a mechanism similar to that which seems to be reasonably adequate for the so-called nonspecific enzymes like NPA. As Dr. Neurath pointed out, there is no direct experimental indication at present that ATEE, for example, is hydrolyzed by ChTr by way of an acyl intermediate.

Dr. H. Gutfreund and I attempted to study this problem by the stopped-flow technique. There is no convenient absorption change when ethyl is the alkyl part of the ester grouping such as there is in the case of NPA. But if acylation occurs with ATEE, there should at first be no liberation of hydrogen ions, whereas during the subsequent deacylation hydrogen ions would be liberated. We therefore hoped that with an indicator in the reaction mixture we might see a lag in the liberation of protons. However, within a maximum of 2 mseconds the liberation of protons was proceeding at its steady-state rate. If acylation takes place, it reaches its steady state in something less than a couple of milliseconds.

Recently Dr. T. Spencer and I have obtained indirect indication as to whether acylation takes place with the specific substrate by some inhibition studies. First we investigated the inhibition of ATEE hydrolysis by NPA. The apparent inhibition constant was very closely the same as the apparent Michaelis-Menten constant for NPA, the quantity that Dr. Neurath called the "deacylation Michaelis-Menten constant." It is well known that, in the

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[4] D. E. Koshland, Jr., Brookhaven National Laboratory.
[5] P. P. Cohen, University of Wisconsin.
[6] M. L. Bender, Illinois Institute of Technology.
[7] G. P. Hess, Cornell University.
[8] J. M. Sturtevant, Yale University.

absence of complications, the inhibition constant pertaining to an inhibitor that is also a substrate should be equal to the Michaelis-Menten constant for that substance if the inhibition is competitive. We thus conclude that NPA is a competitive inhibitor for ATEE and exerts its inhibitory effect primarily by successfully competing for the acylation site on the enzyme. This evidence has somewhat more significance than the well-known observation that acetyl—ChTr does not hydrolyze ATEE.

We also looked at the inhibition of NPA hydrolysis by ATEE. This is considerably more difficult. We have to use large amounts of enzyme to get a large enough change in optical density during the initial burst, and, under these circumstances, when some ATEE is added it is all gone in 5 or 10 seconds. This requires that initial rates be obtained within a few hundredths or a few tenths of a second. Our measurements show that ATEE inhibits, probably competitively, acylation of the enzyme by NPA. This suggests that the two substrates thus compete for the binding site of the enzyme, as well as for the acylation site.

NEURATH: At what *p*H did you carry out the competition studies?

STURTEVANT: At *p*H 8.

NEURATH: I am not sufficiently familiar with the experiments, but it seems to me that there are two possibilities. Either *p*-NPA is hydrolyzed, in which case the nitrophenyl group could fit right into the region on the enzyme normally occupied by the phenyl group of specific substrates such as ATEE, or *p*-NPA inhibits because it is an acylating agent and the acetyl enzyme will not react with ATEE. Were you able to differentiate between the two possibilities?

STURTEVANT: Your latter possibility is correct because the inhibition constant is equal to the apparent Michaelis-Menten constant for NPA, which is 100 times as large as the "true" Michaelis-Menten constant.

This matter of Michaelis-Menten constants brings up another point. I should like to introduce some cautionary remarks with respect to the interpretation of K_m values. Dr. Neurath suggested that these constants can be, in the ChTr and trypsin cases, quite satisfactorily identified in most instances with the dissociation constants of enzyme—substrate complexes. The three-step mechanism, which applies at least to the hydrolysis of NPA, is shown in figure 4:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES)' \qquad (ES)' \xrightarrow{k_2} (ES)'' + P' \qquad (ES)'' \xrightarrow{k_3} E + P''$$

Figure 4

where (ES)″ is acyl—enzyme. According to this mechanism, the true K_m will have its usual definition, but what Dr. Neurath called the K_m for deacylation, which I prefer to call K_m apparent, will have the value $K_m(\text{app}) = K_m[k_3/(k_2 + k_3)]$. Here we are getting quite far away from a true equilibrium constant, and we have to be very cautious in interpreting apparent over-all K_m values as equilibrium constants. In particular, if rate limitation occurs at the k_2 step, the apparent K_m will be equal to the true K_m; whereas if rate limitation occurs at the k_3 step, the apparent K_m will be much smaller than the true K_m. Presumably, much of the difference between the large K_m noted by Dr. Neurath for acetyltyrosinamide and the small value for ATEE can be accounted for in this way.

It should be added that, even in these cases, we may well have additional steps with rate constants k_4, k_5, . . ., and that the apparent K_m will then be given by a much more complicated expression.

NEURATH: I think I have taken care in pointing out that the argument we developed did not apply to ATEE.

STURTEVANT: I have one further point I should like to make. The effect of *p*H on the rates of ChTr- and trypsin-catalyzed reactions is usually taken to indicate that histidine is present in the catalytic site. Additional indication supporting this view derives from the results obtained by Dr. Inagami in our laboratory. He has studied the trypsin-catalyzed hydrolysis of benzoyl-arginine ethyl ester (BAEE) in water–dioxane mixtures and found that the apparent *pK* for the rate-controlling group

hanges by only 0.4 pK unit between water nd 88% dioxane (dielectric constant 7). his strongly suggests that the ionization nvolved is isoelectric, that is, that there s no charge separation. This, of course, is characteristic of the imidazolyl groups.

BRUICE[9]: Unfortunately for the organic hemist interested in the mechanism of steratic enzymes, we do not have cofactors ve can study independently. So we have o follow the literature and guess what unctional groups might be involved. We hought that histidine might be, and re-ently our research has been directed oward determining the efficiency of the midazolyl group as a nucleophilic catalyst or ester and amide hydrolysis. If the en-yme binds the substrate and an imidazolyl roup is responsible for the displacement eaction, a configuration something like igure 5A might occur. This is an intra-omplex reaction and can be duplicated with an intramolecular model where the methylene bridge replaces enzyme (fig. 5B). When X = p-nitrophenolate, a rate

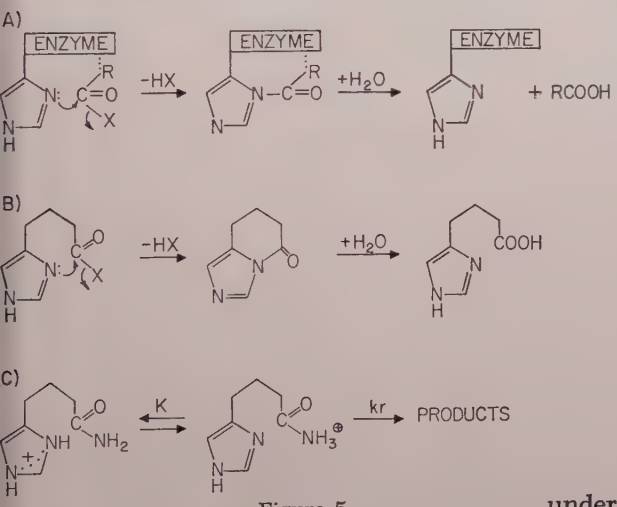


Figure 5

onstant (25°C.) of 232 min^{-1} was ob-ained, this being actually greater than hat determined by Dr. Sturtevant for he ChTr—p-NPA complex (185 min^{-1} 25°C.). As we go down the series from X = m-nitrophenoxide, p-chlorophenoxide o X = phenoxide, the rate drops drastic-lly. However, even the slowest (7 min^{-1}) s in the enzymic range, being for instance greater than the rate of hydrolysis of phenyl acetate by wheat germ lipase. When X = n-propylthiol, the rate was about that of X = phenoxide or some 10^6 as large as that of a normal thiol ester.

When we came to X = OCH_3, we found there was no imidazole catalysis. However, I do not wish to leave the impression that imidazole will not catalyze the hydrolysis of aliphatic esters, for it does participate in the hydrolysis of 4-(2′-acetoxyethyl) imidazole. When X = NH_3, imidazole acts as a catalyst, but the mechanism is different from that of the esters and occurs by preequilibration of the imidazolium species with the amide bond (fig. 5C). The rate of the amide hydrolysis was observed at 78°C. and is certainly not in the enzymic range.

In summary, we can say that, for the phenolic and thiol esters, all that would be needed for an enzymic rate of the ester bond-breaking process would be the absorption of the ester on a protein at the site of histidine so that the steric relation of the imidazolyl group to the ester bond would be as in our model. This is not to say that imidazole is involved in any enzymic reaction; these are simply the facts as found in the organic laboratory and indicate the possibilities.

It might be suggested that the amide and methyl ester could be made to react at enzymic rates if there were more steric compression of the imidazole against the ester or amide bonds. Models providing this compression are now under study.

VISWANATHA[10]: We have attempted to obtain a small active fragment or derivative of the enzyme trypsin that might help in understanding the interrelation among catalytic activity and the secondary and primary structures in the enzyme protein. We started with the inactive protein precursor, trypsinogen, to avoid the possibility of activity in degraded preparations being caused by the starting material itself.

[9] T. C. Bruice, Johns Hopkins School of Medicine.

[10] T. Viswanatha, National Institutes of Health, Bethesda.

Thanks to the work of Dr. Neurath and his associates, we know the partial sequence of trypsinogen, which can be written as follows: Val—(asp)$_4$—lys—Ileu. During the activation of this zymogen by trypsin, one peptide bond is hydrolyzed, the hexapeptide Val—(asp)$_4$—lys comes off, and the otherwise inactive zymogen becomes enzymically active trypsin.

When we started with trypsinogen, it was virtually inactive and to remove all possible traces of trypsin and ChTr the zymogen was treated with DFP. The DFP-treated trypsinogen was acetylated with acetic anhydride in the presence of 0.5 saturated sodium acetate at 0°C. During this acetylation step, the free amino groups on the protein are blocked. The acetylation step was repeated twice to block as many amino groups as possible.

The twice-acetylated trypsinogen has nearly 90% of the original amino groups blocked and is inactive when tested against the substrate BAEE. Attempts to activate the acetylzymogen with either trypsin or ChTr in the presence or absence of 3.0 *M* urea were unsuccessful.

We then tried pepsin as an activating agent. The peptic activation had to be carried out at *p*H 3.2, where pepsin can still function and denaturation of acetyltrypsinogen is minimized. When pepsin was used under these optimal condition to our surprise, activation of acetyltry sinogen was observed. The type or resul obtained is shown in figure 6. The acety trypsinogen, which is inactive to start wit becomes active on degradation by pepsi and, once the maximum activity is o tained, it stays constant throughout th subsequent degradation. During this pe tic activation, acetyltrypsinogen, which insoluble in 3.6% trichloroacetic ac (TCA), is rendered soluble. Maximu activity is achieved when about 40% the protein becomes soluble, correspondi to the cleavage of five to six peptide bon in the protein; and once this maximu activity is obtained, further cleavage ca occur without loss of this activity. Near 90% of the acetylzymogen becomes solub in TCA in 18 hours and pepsin is restricte to its specificity requiremnets during th period. But, the last 10% solubility is ve slow to achieve. This can be accelerate by raising the temperature to 30°C., b this temperature leads to inactivation the active enzyme that formed. So v keep the temperature of activation at 2 26°C. When 90% solubility in TCA is o tained, more pepsin is added and the sol tion is allowed to incubate until 100 solubility in TCA is achieved. Usually th process takes 24–40 hours. We treat t

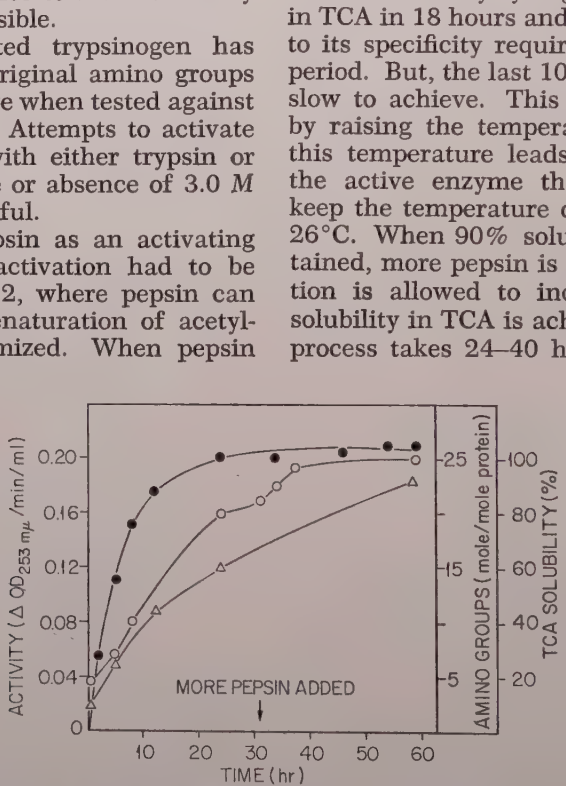


Fig. 6 Activity (●), release of amino groups (△), and solubility in 3.6% TCA (○) accompanying the action of pepsin on acetyltrypsinogen. A solution of 1.75 g of trypsinogen (50% $MgSO_4$), adjusted to *p*H 7.0, final volume 125 ml, was treated with 100 μl of pure DFP and acetylated twice as previously described. After dialysis, 860 ml of a solution containing 750 mg of protein (85% of the amino groups acetylated) was brought to *p*H 3.2, and treated with 8 mg of pepsin (suspended in 2 ml of 0.01 *N* HCl). Arrow indicates time at which an additional 5 mg of pepsin was added. Digest was allowed to stand at room temperature during which time aliquots were removed for the indicated analyses.

active peptic digest with ammonium sulfate to 0.3 saturation. The precipitate formed represents 10% of the original 280 mμ absorbing residues, the remainder being in the supernatant. All of the activity is quantitatively recovered in the precipitate, the supernatant being totally inert. The precipitate is dissolved in water and the *p*H brought to 7.0 to denature the pepsin present. Then the solution is dialyzed against 0.001 *N* HCl for 3–4 hours. A bulky inactive precipitate is formed and is removed by centrifugation, and the clear supernatant contains all of the activity. At this stage, the preparation has ten times the specific activity of the activation mixture before purification. When this active derivative preparation was subjected to chromatography on DEAE-cellulose column, nearly 70–75% of the protein moved as a single peak containing all of the activity. The inactive contaminant, represented by about 25% of the material, can be easily separated. There is no variation in specific activity from one fraction to another in the active peak. We used the chromatographically pure active derivative preparation in our subsequent studies. Sedimentation studies show a single, homogeneous peak with a $S_{20,w}$ value of 0.9 Svedburg unit. Reaction with fluorodinitrobenzene suggests that phenylalanine is the N-terminal amino acid, and quantitative analyses for the DNP-amino acid gives a molecular weight of 6000 for the active derivative. We studied the reaction of the active derivative with DFP^{32} but have not done the chromatographic separation. However, we still get an estimate of minimum molecular weight of 5500, based on the assumption that 1 mole of phosphorus is incorporated into the protein during the reaction with DFP^{32}.

The specificity of the active derivative is less restricted than that of trypsin; for example, it hydrolyzes ATEE fairly rapidly. This activity is nearly 5% of the trypsin-like activity of the molecule and cannot be attributable to ChTr contamination because we have been able to show that there is not enough ChTr in the starting material to give this amount of activity.

We wanted to know whether the active derivative contained any histidine and therefore made an elaborate study. Trypsinogen has 3.0 moles of histidine per 24,000 g of protein. The peptic digest of acetyltrypsinogen was also found to contain 3.0 moles of this amino acid per 24,000 g of original protein. As this peptic digest is purified, the specific activity of the active derivative increases and histidine is lost. The chromatographically pure material shows a value of 0.14 mole of histidine per 6000 g of protein on microbiological assay. This last trace we have not been able to remove completely; we would like to remove it because it is a very important point. We can also obtain a partially purified active derivative that has 0.44 mole of histidine per 6000 g of protein. The specific activity of this material is much lower than the preparation with 0.14 mole of histidine. But we can improve the activity by further purification, with loss of histidine.

A point I want to make at this stage is that, once the maximum activity is obtained during peptic activation, it does not drop but stays constant throughout the process of peptic action. This observation, coupled with the fact that when we remove histidine the specific activity of the active derivative increases, suggests that histidine may not be essential in this active fragment of trypsin. I do not know if this is true for native trypsin or ChTr, but certainly it is suggested for our active derivative, which is qiute different from trypsin. So we are inclined to think that histidine may have no role in this particular active derivative and that, had there been one histidine associated with the activity, we should have seen at some stage a drop in activity during peptic activation.

NEURATH: When experimental evidence is introduced that seems to be at variance with all previously accepted data, it becomes imperative to examine such evidence; and, in view of the considerable importance of Dr. Viswanatha's findings, with which I have become familiar through his courtesy, it becomes important to examine in more detail the evidence that has been presented to us.

Clearly the conditions under which the active fragment, if the term is permitted, has been obtained are unconventional. I think that the most important points we have to consider are the purity of the

fragment and the criteria for its enzymic activity, because it requires a pure compound to correlate activity with the chemical nature of the product. Even if we are dealing with a fraction that, under the most careful conditions of chromatography appears to be homogeneous, the question remains whether all necessary precautions have been taken to exclude the possible presence of a material that may have escaped enzymic degradation and may be responsible for the observed enzymic activity. For instance, sedimentation analysis would not be conclusive in this respect if the fragment is much smaller than the original molecule or if interaction of the two species occurs.

It would be interesting to know how the maximum activity produced by peptic activation of acetylated trypsinogen compares to that normally achieved by tryptic activation of trypsinogen. If I am not mistaken, the specific activity, on a weight basis, of the purified fragment is one-half that of trypsin toward BAEE, which would mean that the molar turnover number is only one-eighth that of trypsin. I was surprised to note that the fragment Dr. Viswanatha described contained 19 of the 21 glycine residues of trypsinogen, particularly since we know from Dr. G. H. Dixon's degradation and sequence studies in our laboratory that more than two glycine residues occur elsewhere in the molecule. I wonder, too, whether it is a valid argument to compare the activity of various preparations to their histidine content and whether, with the same justification, a correlation could be established with other amino acids that we may care to choose. The point that there is at least 0.2 equivalent of histidine per mole of fragment cannot be ignored, and I think it would be important to know whether it is there as part of the molecule or as part of an impurity that is the carrier of enzymic activity.

Lastly, the question of the increased range of specificity of the fragment as compared to trypsin perhaps should demand more attention than Dr. Viswanatha has indicated, because it raises the question, in my mind at least, of wheth these changes in specificity could be e pected if ChTr or some other pancreat proteolytic enzyme were concentrated this fraction by a process of degradati of denatured material.

I am compelled to raise these questio because it seems to me that this is e tremely important work, and I have e couraged Dr. Viswanatha on previous o casions to continue these studies with pa ticular emphasis on the question of th purity and homogeneity of the fragmen both enzymically and chemically. Obv ously, for this evidence to stand the te of time, it is necessary that the fragme be homogeneous by the most vigoro enzymic and chemical criteria.

VISWANATHA: As regards the conditio under which the active derivative is o tained, we are limited in our choice experimental conditions. We carry ou peptic activation at *p*H 3.2, which is o timum inasmuch as the denaturation the acetylzymogen is minimized and pe sin can still function at this *p*H. I agre with Dr. Neurath that this is indeed a ve unusual case of activation.

Dr. Neurath raised the possibility tha the activity is associated with the fra ment that might arise from the nativ enzyme. I can say that we take eve precaution to start with enzymically ine zymogen, and the acetylated zymogen b fore peptic activation is completely devoi of catalytic activity. If there is any co taminant activity, we should be able t detect it. Indeed, one of the prerequisite for obtaining a nice preparation of th active derivative is that the starting tryp sinogen be trypsin free. Even a slight co tamination in the starting material lead to unsatisfactory results.

As to the question about activity mea urements, all determinations were mad under identical conditions throughout th period of activation. Comparison betwee the active derivative and trypsin or acety trypsin is not strictly valid since we ar dealing with different systems. But, if w were to make such a comparison, th information obtained would depend o the nature of the substrate. Thus, usin BAEE, we find the active derivative i

half as active as trypsin on a weight basis. A most significant experiment on this point involves the use of oxidized insulin as a substrate. With this material, we find that the active derivative is more active than trypsin in that it cleaves seven to eight peptide bonds as compared to two peptide bonds cleaved by trypsin.

Dr. Neurath, by mistake, stated that the active derivative fails to catalyze the hydrolysis of *p*-NPA. Actually, the active derivative catalyzes the hydrolysis of *p*-NPA but does not give the initial burst of 1 mole of *p*-nitrophenol as is the case in similar experiments with ChTr or trypsin. I do not know the reason for this, but I can say that even this ability to catalyze the hydrolysis of NPA is destroyed when the active derivative is treated with DFP. Even with native trypsin, we observed anomalous results with this type of study. The initial burst of 1 mole of *p*-nitrophenol is observed with trypsin only in phosphate buffer and is not found if the buffer system is changed to either borate or Tris, the other reaction conditions being the same.

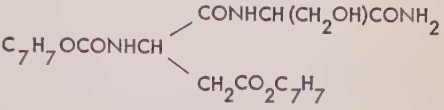
Finally, I would like to comment on the amino acid content of the active derivative. Dr. Neurath wondered about the large number of glycine residues in it. As I had mentioned to him, the glycine and alanine peaks in the amino acid chromatogram overlapped considerably. By trying to resolve the individual components of this superimposed peak of glycine and alanine—a calculation that cannot be done without serious error—we got the high value for glycine, which certainly is not a true picture.

I wish to take this opportunity to thank Dr. Neurath for his kindness and valuable suggestions.

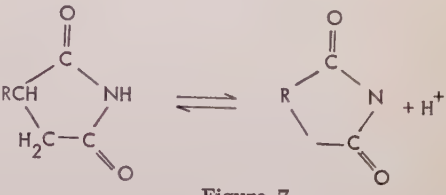
BERNHARD: I would like to summarize some work done in collaboration with my colleagues Drs. E. Katchalski and M. Sela and particularly Dr. A. Berger at the Weizmann Institute in Israel.

As a result of a communication from Dr. Viswanatha's laboratory, we were particularly interested in the chemical behavior of peptides of the type that Dr. J. A. Cohen spoke about (Cohen *et al.*, fig. 6, this Symposium).

We first prepared the benzyl ester of the model peptide of aspartyl serine blocked at either end by peptide bonds (I; fig. 7).



(I)



(II)

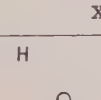
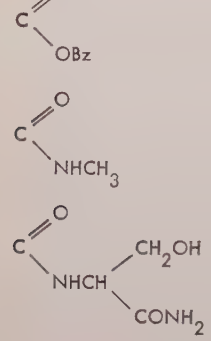
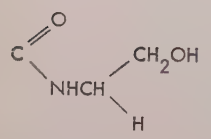
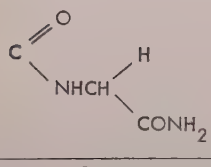
Figure 7

From I, we wished to prepare the corresponding β-carboxylate (i.e., the smallest peptide model of the aspartyl-serine sequence) by hydrolysis. Benzyl esters are relatively stable and not susceptible to general base catalysis. To our surprise, we found that, even at what corresponds to *p*H 7, this benzyl group was quite rapidly eliminated (the [OH] was approximately 10^{-7} *M* in 50% aqueous dioxane), with a half life of about 30 minutes at *p*H 7, 1½ minutes at *p*H 8, and about 0.10 minutes at *p*H 9. Relative to benzyl propionate, the rate of hydrolysis of this compound is $\sim 10^5$ to 10^6 times as fast.

It was of interest to determine the structural features responsible for such a tremendous activation at the β-carboxyl. We prepared a number of derivatives. In table 10 the rates of hydrolysis relative to benzyl propionate are listed. We found that with no peptide group at all at the α position the rate was essentially the same as that for benzyl propionate, but as soon as a peptide bond was made at the α-carboxyl (compounds 3, 5, and 6) the rate went up by a factor of 1000. It did not matter whether this peptide group was a methyl group, an ethanolamine group, or another amino acid amide (other than serinamide). Any compound with a peptide bond increased the rate by a factor of almost 1000 over ordinary benzyl esters, but it was still slower by a factor of nearly 1000 than when R was serinamide.

TABLE 10

Relative rates of hydrolysis of benzyl esters of CbzoNH CH ($CH_2CO_2C_7H_7$)—X in 50% aqueous dioxane

	X	V_0 relative
1.	H	3
2.		1
3.		10^3
4.		10^5–10^6
5.		10^3
6.		10^3

[a] Approximate values since the peptide reactions are not first order in OH^-. All values are adjusted to $[OH^-] \approx 10^{-5}$ *M*.

The primary α-amide of *N*-carbobenzoxyaspartic acid β-benzyl ester was prepared and subjected to these same conditions for basic hydrolysis. Rather than hydrolysis, cyclization with the elimination of benzyl alcohol formed an imide structure (II; fig. 7), which was the only product of the reaction. The acid form of the imide was isolated, and the specific optical rotation was found to be −45°, the specific rotation of the initial reactant was +2°. The imide dissociates with *p*K 9.5 to give N^-. The presence of the negative charge makes the compound resistant to further hydroxyl-catalyzed degradation (to form the β-carboxylate). If, instead of the primary NH_2 we had NHR, cyclization would no longer result in an ionizable imide. Hence secondary imides should be more susceptible to base hydrolysis than primary imides.

If intermediates were forming in the case of the secondary amides (or peptides), we should be able to detect them by following the optical rotation as a function of time. We have done this with compound 3, table 10. This compound did not exhibit much rotation ($\alpha_d = +2°$). Incubation of the reactant at a *p*H at which hydrolysis could be observed resulted first in a rapidly rising rotation and then in a gradual return to nearly zero rotation, indicating the transient formation of a cyclic intermediate. From these data we determined the molar rotation of the intermediate, which corresponded to that determined for the primary imide. When the optical rotation of the serine derivative (I) was followed in this way, similar intermediate was formed. The rate of formation was five times as fast as that of compound 3. The rate of hydrolysis of the intermediate, however, was about 100 times as fast as that of compound 3. The molar optical rotation change in forming the intermediate from I was considerably greater (more negative).

The general mechanism for hydrolysis of β-esters of aspartyl peptides is the pathway shown in equation (3) (fig. 8). We

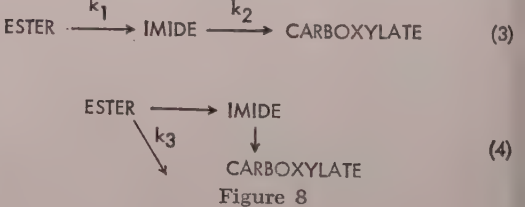


Figure 8

could fit the data for the hydrolysis of compound 3 (which we measured with the *p*H stat) to the over-all data measured polarimetrically with these two kinetic constants ($k_1 > k_2$). With serinamide peptide, however, we had to assume, in addition, an unstable intermediate via which carboxylate was directly formed ($k_3 > k_1 > k_2$) [eq. (4) fig. 8].

These facts fit together to define a precise geometrical model of the catalysis. The only function we could ascribe to the terminal amide bond and the initial acyl (peptide) bonds in this molecule (I) was

a structural one. We assumed that somehow this held the reactive structure together. With the aid of space-filling peptide models, made according to the criteria of L. Pauling and R. B. Corey, we constructed the molecules. We made the imide ring first and found that, when a hydrogen bond from terminal N to initial O (carbonyl) was made, the oxygen of the serine hydroxyl was at the β-carbonyl of what once was the β-carboxyl of aspartic acid. This is a rigid structure as is shown in figure 9. After the hydrogen bond and the imide ring are made, the seryl hydroxyl must be at this β-carbonyl. The structure of the resultant intermediate is shown in figure 9; i.e., an oxazolidine ring that is fused to what once was an imide ring.

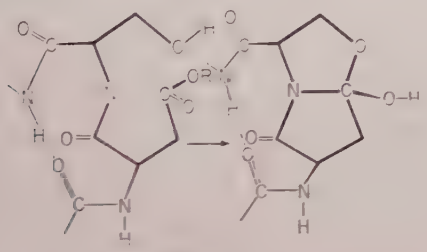


Figure 9

There is no reason (from our work) why a similar reaction should not proceed if the benzyl ester were replaced by $CONH_2$ in which case the structure of the original peptide would be R^1—asparaginyl—seryl—R^2. An intermediate ring of this type (fig. 9) might have a *pK* in the region of neutrality and might explain the *p*H dependence of the enzyme. I think Dr. Brenner's work, which implies that a pK^1 around neutrality for a structure of this type is reasonable, substantiates this to some extent.

I should like to show how such an intermediate could function in the catalysis of ester hydrolysis. In the case of a strong ester bond, we would form a tetrahedral intermediate that would attack at the carbonyl carbon (fig. 10). The strength of the attack (the effectiveness of catalysis) would be a function of the polarizability of the C—X bond (fig. 10). This is in keeping with Dr. Neurath's data. We know, through work of Drs.

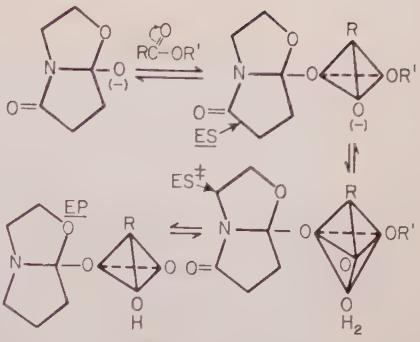


Figure 10

Bender, Koshland, and me, that such enzymes do seem to have a site for the water molecule. From the tetrahedral configuration shown, a Walden inversion-type mechanism leads (in the case of true substrates) to the enzyme-product complex, which in a sense is an inversion of the configuration. The final step is the dissociation of the enzyme-product complex. It would dissociate rather easily for carboxylate products and may be the reason products of proteolytic reactions dissociate from the enzyme site.

We tried to build the model of enzyme catalysis proposed by Porter *et al.* ('58) and Rydon ('58) and could not build it because we cannot go from one ring intermediate to another since the bonds point in opposite directions. This is the only good reason I know of for excluding Rydon's mechanism. We can, however, build ours with the Cal Tech models.

Figure 11 illustrates what might happen if the C—X bond were very weak. Cleavage of this bond without simultaneous attack by another group leads to an

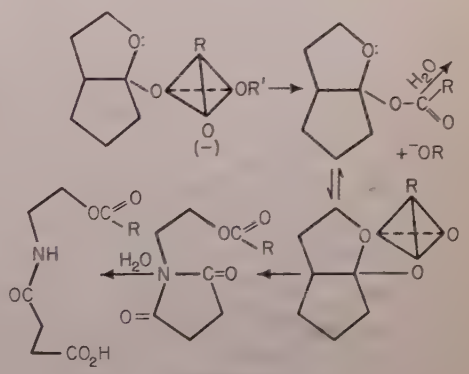


Figure 11

active acyl—enzyme. Whether or not this type of compound forms would depend on the strength of the C—X bond. A weak bond like C—OR in NPA might form this acyl derivative. An intermediate of this sort could either undergo hydrolysis of the acyl group to regenerate an active enzyme, in which case inhibition would not be very effective, or could form an acetyl ester through a tetrahedral intermediate involving the oxygen from the serine ring. The transfer of the acyl group from one oxygen (the labile derivative) to the other (the stable serine ester) may be the mechanism for the oft-noted changes observed on aging of acetylated and phosphorylated enzymes. This serine ester would now be a compound similar to compound 3 (table 10). After sufficient time at *p*H 8, the imide ring would finally open up, and the whole structure might unfold (fig. 11). Opening of the ring structures should result in loss of optical rotary power from three centers, viz., the rigid asymmetric α-carbons of the aspartyl and seryl rings and the asymmetric bridgehead carbon from the β-carboxyl of aspartic. This should result in an optical rotation change (calculated) of a few degrees and is in keeping with that observed after inhibition by DFP.

STURTEVANT: The mechanism proposed by Dr. Bernhard seems to involve an ionization with a charge separation, which should be more affected by changes in dielectric constant than the ionization in the case of trypsin is observed to be.

BERNHARD: I think that, if there are charged peptide species (as there are here), then the "effective dielectric constant" is more the result of the proximity of the charges than of the dielectric constant of the medium. If these charges are rather close together, I think we can almost forget about the dielectric constant of the medium.

STURTEVANT: I do not quite understand how your mechanism accounts for a *p*H control of the final deacylation. I may have overlooked that.

BERNHARD: If the proton is at the bridgehead nitrogen, the structures shown in figure 8 cannot be written so that the mechanism can proceed to EP.

J. A. COHEN[11]: I should like to ask Dr. Bernhard what happens to his scheme when he gets the glutamic rather than aspartic acid in the active site.

BERNHARD: I am sorry I did not know about the glutamic acid case when we were making the models. You certainly could form a six-membered imide ring. Formation of a six-membered ring is much less probable than formation of five-membered rings. As to the stability of an already formed six-membered ring, I think it should be at least as stable in the bicyclic structure. Six-membered rings are much harder to form. Maybe that is why the pseudoesterases are not quite so good as the real thing.

VISWANATHA: Dr. Cohen, since you have commented on the same sequence with quite a few enzymes, would you like to comment on the specificity of the enzymes; i.e., whether the enzyme has the same active site as the one that binds the phosphate and how it is taken out?

COHEN: I think that we should not be dogmatic about the term "active site." The B group is probably only part of the active site; we think it is a common denominator that is part of the active site and enzymic activity and substrate specificity are conveyed to the enzyme molecule by quite a number of additional secondary and tertiary structures.

VISWANATHA: I would like to explain the change in specificity I was talking about since we have a similar active site. The experiment is still in progress. We are trying to find the place of the combined site in the active fragment, and I should like to speculate that, as we go on with the secondary structure, maybe we will get the enzyme to function on different peptide bonds. This work is with oxidized insulin.

BINKLEY[12]: It is necessary every once in a while to try to reconcile all fields at the same time, so I thought I would bring peptidases into the same story. There are, as we have mentioned, two types of peptidases. The first type is readily soluble and labile to proteolysis. Another type of

[11] J. A. Cohen, Medical Biological Laboratory, Rijswijk.

[12] Francis Binkley, Emory University.

peptidase is insoluble and stable to proteolytic digestion.

The labile types can be extracted easily from any tissue and can be digested with ChTr without loss of activity. Leucinamino peptidase (LAPase) can be digested with ChTr to give a small fragment. The digestion, with the release of the active fragment, increases the specific activity from a C_1 of ~100 to a C_1 of ~400. This on a total nitrogen basis. Soluble LAPase does not react with DFP, but it does have a peculiarity that may explain this. If soluble LAPase is hydrolyzed with 1 *N* acid for 30 minutes, a ninhydrin-reactive material will be released. In the material ordinarily isolated, this ninhydrin-reactive material may be any amino acid. If, however, LAPase is digested with leucinamide, leucine is the predominant labile amino acid. So it would seem that the active form of LAPase is acylated, and the acyl grouping may stand in the way of the addition of the DFP. The soluble LAPase is destroyed very quickly by digestion with trypsin but not with ChTr. Therefore, tryptic digestion together with the acyl grouping may place the soluble LAPase in the same category as the other hydrolyzing enzymes and, of course, the acylation may be a stabilizing factor and there may be a form in which acyl and DFP will react.

The insoluble peptidases are those resistant to digestion by trypsin, ChTr, or any other proteolytic enzyme. If the insoluble material is released by a short digestion with trypsin, a compound can be isolated that has approximately nine stable (or peptide-linked) amino acids to one acid-labile amino acid. The ratio of absorption, 260:280, is ~0.8. In other words, this is quite a respectable looking protein-type enzyme and still has acid-labile amino acids.

If the solution is thoroughly digested with ChTr and trypsin and reisolated on an ECTEOLA column, the ratio of labile and stable amino acids is 1:1. These acid-labile amino acids cannot be in the peptide form. The C_1 value with leucine is ~2000. So we are not dealing with lower activity; we are dealing with extremely high activity.

The resistant peptidases contain guanine nucleotides as well as the labile amino acids. It may be possible that, at the high *p*H optimum of these enzymes (they are essentially inactive at neutral *p*H values), the guanine may substitute for the imidazole grouping and the hydroxyl groupings of the ribose may serve the same role as the serine hydroxyl. These peptidases do not contain histidine.

BERNHARD: If you call the five-membered ring in guanidine "imidazole" then, as Drs. Bruice and Bender and others have shown, the anticipated velocity of such reaction is dependent on the base strength and this is an extremely weak base. Of the two purine constituents this is the worse.

What is more important and disturbing in regard to this (and other) models, is that the true substrates of these enzymes are not subject to "general base catalysis." To postulate a mechanism of this sort, we have to show something specific about the mode of hydrolysis of these compounds because the general base catalysis just will not do.

BINKLEY: Just one thing we have not pointed out: this is a zinc-containing complex, which may have a little to do with the mode of action.

NEURATH: I think that, within the context of this discussion, it might be well to recall that several of the enzymes occur in nature as precursors or catalytically inactive proteins and that the opening of one and only one peptide bond suffices to convert the parent zymogen into active ChTr or trypsinogen. We do not know exactly what the concomitant structural and configurational changes are, but we do know that they occur, as manifested by measurements of optical rotation and other means.

It seems to me that any mechanism purporting to explain on a model basis enzymic activity must take into consideration changes that apparently bring distant groups into proximity. Arguing from this point, which I do not want to belabor, it seems to me that the evidence based on these findings, as well as on the effects of denaturation on the reaction of these enzymes with acylating or phosphorylating agents, clearly indicates that tertiary fold-

ing is an important factor in the catalytic function of the enzymes.

BERNHARD: I should like to show the geometry of the hydrogen-bonded model. To have the proper geometry for formation of the bicyclic structure, we have to make a specific hydrogen bond. To hold together this somewhat strained ring intermediate, it is obvious that the two peptide chains coming in and out in the enzyme sequence contribute more to the real structure of the enzyme than just one hydrogen bond. I think it is for this reason that in this model compound it is merely an unstable intermediate, whereas in the enzyme it might become stable if the added hydrogen bonds of the peptide contributed to specifying this geometrical arrangement.

BENDER: Dr. Bernhard's model is essentially a tetrahedral intermediate that is stabilized in a peptide sequence. As such it should be rather high energy, in the sense of the biochemist's usage, and possibly has merit to it.

I should like to describe another enzyme model not containing imidazole that Dr. G. Schonbaum and I investigated. Based on papain, it exhibits some interesting organic chemistry. We have investigated the hydrolysis of *p*-nitrophenyl acetate in the presence of thiosalicylic acid. Thiosalicylic acid contains a sulfhydryl group of *pK* 8.4 and a carboxyl group of *pK* around 4.5, fortuitously close to the *pK's* of the groups presumably involved in papain action.

We have a simple example of a sulfhydryl group and a carboxyl group adjacent to each other in a rigid configuration. There is an initial complex formation between *p*-NPA and thiosalicylic acid. At *p*H's around 7 there is a facile formation of *p*-nitrophenoxide ion; this reaction involves the dianion of thiosalicylic acid and forms a thioester. The thioester is a simple analog of aspirin, namely thioaspirin. The hydrolytic rate of thioaspirin is faster than that of aspirin. The mechanism of this process should be similar to that of aspirin; that is, the intramolecular formation of a mixed anhydride that spontaneously decomposes to regenerate thiosalicylate and produce acetic acid.

The over-all sequence then involves co plex formation, an intermolecular reacti by a good nucleophile (S^-), and an intr molecular reaction completing the ca lytic process.

MOUNTER[13]: The irradiation of ChTr i activation can be measured by either pr teolytic or esterase assay, or by usi DFP[32] to label the specific sites capab of reacting. A linear inactivation cur is found in each case but there is a di ference. We found that the proteolyt falls off far more rapidly, the esteras less rapidly, and the P^{32} labeling least. W have now extended this in preliminar experiments to the cholinesterases, whe we are finding similar differences wit acetyl-, propionyl-, and butyrylcholine substrates. There is only partial destru tion of the enzyme, but certainly there creation of some form of disorder by i radiation that is modifying kinetics. will not go farther than that.

[13] L. A. Mounter, Medical College of Virgini

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ıe Chemical Structure of Chymotrypsin

BRIAN S. HARTLEY
Department of Biochemistry, University of Washington, Seattle

Chymotrypsinogen is a protein that can isolated in a high degree of purity. ce its composition, properties, and ivation to chymotrypsin (ChTr) have eady been reviewed (Neurath, '57; Neu- h *et al.*, '59), I shall only summarize information.

The zymogen is a single peptide chain of lecular weight 25,000 with an N- minal half-cystine residue and a C- minal asparagine. The amino acid com- sition (Wilcox *et al.*, '57) shown in table may be treated with considerable con- ence, and no evidence exists that groups ıer than these residues are present. ere are five disulfide bridges and no free SH groups. When chymotrypsinogen is ubated with trypsin, the activity of Tr appears, coincident with the split- g of an arginyl bond in the sequence

—Leu↓—Ser.Arg↓—Ileu.Val—.

The leucyl bond in this sequence is then split autocatalytically, yielding δ-ChTr, which has two peptide chains, and liberating the dipeptide Ser.Arg. Further autolysis, with liberation of a dipeptide Thr.Asp —NH_2, may occur to yield the common form, α-ChTr, which has three chains. Other forms of ChTr, derived from degraded chymotrypsinogens (the so-called neochymotrypsinogens) in which other peptide bonds have been split have been reported (Rovery *et al.*, '57). It might be emphasized, however, that in all cases activation coincides with splitting of the —Arg. Ileu— bond, and the various ChTr's differ only slightly in activity.

Meedom ('56a,b) separated the three chains of α-ChTr after performic oxidation of the disulfide bridges. End-group analysis of the three chains enabled their order in the zymogen to be determined. Figure

TABLE 1
Distribution of amino acids in α-chymotrypsin

Amino acid	Residues/mole						
				B Chain			C Chain (est.)
	Chymotrypsinogen	Activation peptides	A Chain	Total	"Core" (est.)	"Soluble peptides"	
½ Cys	10	–	1	7	6	1	2
Asp	22	1	–	20	12	8	1–2
Thr	23	1	–	17	12	5	5
Ser	30	1	1	28	21	7	1–2
Glu	14	–	1	12	7	5	1
Pro	9	–	2	6	4	2	1
Gly	23	–	2	16	14	2	5
Ala	22	–	1	18	9	9	2–3
Val	22	–	2	16	14	2	4
Meth	2	–	–	1	1	–	1
Ileu	10	–	1	7	5	2	2
Leu	19	–	2	15	8	7	2
Tyr	4	–	–	3	1	2	1
Phe	6	–	–	6	3	3	–
Lys	13	–	–	11	1	10	2
His	2	–	–	2	2	–	–
Arg	4	1	–	2	–	2	1
Try	7	–	–	6	5	1	1
Amide	25	1	1	?	?	9	?
Total	242	4	13	193	~125	68	~34

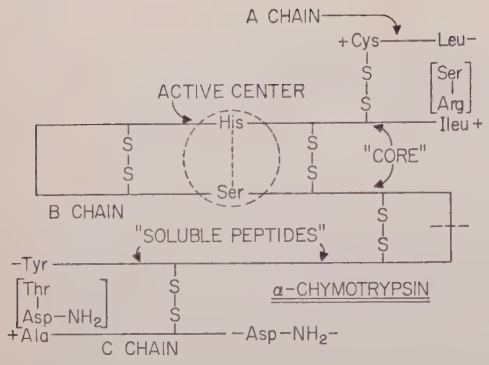

Figure 1

1 is a diagrammatic representation of α-ChTr showing the order and approximate relative lengths of the three chains and the origin of the two peptides released on activation.

The A chain is N-terminal in the zymogen and contains only 13 amino acid residues; Meedom ('58) determined their sequence.

$CySO_3H$.Gly.Val.(Ala,Pro).Ileu.Val.Pro.Glu—NH_2.Leu.Ser.Gly.Leu

The C chain is also small [Meedom ('56b) reported 50 residues] and occupies the C-terminal position in chymotrypsinogen.

The B chain represents 80% of the molecule and has been studied after isolation from performic-oxidized α-ChTr (Hartley, '58a). Its amino acid composition (Hartley, '59) is shown in table 1, giving a molecular weight of around 20,000. Both histidine residues occur in this chain, as does the unique serine residue that reacts with DFP (Meedom, '56a), and hence the elements of the "activating site" are found here.

Digestion of this oxidized B chain with ChTr yields a large, relatively insoluble peptide "core" and a series of small "soluble peptides" (Hartley, '58a,b, '59). The amino acid sequences of the latter are shown in table 2. Some of these peptides are clearly derived from their fellows, and the nine starred peptides represent unique sequences accounting for 68 of the 146 residues in the chain. None of the soluble peptides contains an N-terminal Ileu.Val sequence that would be characteristic of the N-terminal end of the whole chain, and we may therefore ascribe them to the C-terminal end. On this assumption, the core would represent the N-terminal portion of the B chain.

We are therefore in a position to discuss the distribution of amino acids between the A chain (13 residues), the core (~ 76 residues N-terminal in the B chain), the soluble peptides (~ 70 residues C-terminal in the B chain), and the C chain (~ 50 residues).

We find (table 1) that the soluble peptides contain 10 of the 13 lysine residues

TABLE 2

Soluble peptides from oxidized B chain

1 A	Large peptide, possibly from "core," containing $CySO_3H$,Lys,Asp,Ser,Glu,Ala, Val,Leu,Tyr
1 B	Ala.Asp—NH_2.Thr.Pro.Asp.Arg— Leu.Glu—NH_2.Glu—NH_2.Ala.Ser.Leu
*1 C	Leu.Glu—NH_2.Lys.Ser.Gly.Glu.Asp.Ser.Lys.Ileu
*1 D	[Asp,Glu,(—NH_2)].Lys.(Gly,Thr).Phe
*1 E	Ala.Ala.Asp—NH_2
*1 F	Ala.Asp—NH_2.Thr.Pro.Asp.Arg— Leu.Glu—NH_2.Glu—NH_2.Ala.Ser.Leu.Pro.Leu
1 G	Ser.(Thr.Ala),(Ala,Glu,Leu,OxTry),Ser.Phe
*2 A	(Asp—NH_2,Ser,Leu).Thr.Asp—NH_2.$CySO_3H$.Lys.Lys.Tyr
*2 B	Lys.Leu.Ser.(Thr,Ala).Ala.Ser.Phe
3 A	Lys.Asp.Ser.Lys.Tyr
*3 B	Ala.Arg.Val.Thr.Ala.Leu
*4 D	Lys.(Ileu,Ala).Lys.Val.Phe
4 E	Lys.Leu
*5 A	Lys.Asp—NH_2.Ser.Lys.Tyr

d two of the three arginine residues of
e enzyme, but only four free acidic
ɔups. This small area must, therefore,
ntain most of the positive charges in the
ɔlecule. The remaining arginine and two
the three remaining lysine residues are
parently in the C chain, but both histi-
ne residues are found in the core, which
esumably also contains the sequence
ɔund the unique "active serine residue."
ence the elements of the activating site
e present in this part of the molecule,
nich is remarkably deficient in basic
nino acids and contains sufficient cystine
d proline residues to allow deviations
ɔm an α-helix.

Present studies of the sequence in these
ɔteins are focused on *S*-sulfochymotryp-
ɔgen, where the disulfide bridges have
en broken by reduction and the trypto-
an residues remain intact (Pechère *et*
, '58). Tryptic digestion of this single
ain has yielded several fairly small pep-
es plus a core resembling in many ways
at derived from the oxidized B chain.
is is consistent with the foregoing ideas
out the distribution of basic residues,
ace the oxidized core would possess only
e bond susceptible to splitting by tryp-
. Studies of the composition and se-
ence of these peptides are in active prog-
ss, but some preliminary results may be
interest here.

One of the two histidine residues in the
re occurs in the sequence Ala.His.Phe,
d the other is found in a larger peptide-
ntaining cystine. It may be possible, by
action with bromoacetic acid, to distin-
ish which of these residues forms part of
e activating site (Stein and Barnard,
). Further preliminary results indicate
at the following sequence occurs at the
erminal end of the B chain:

ys.Leu.Lys.(Ileu,Ala).Lys.Val.Phe.Lys.—
sp-NH_2.Ser.Lys.Tyr.Thr.Asp-NH_2.Ala.Asp-NH_2.—
hr.Pro.Asp.Arg.Leu.Glu-NH_2.Glu-NH_2.Ala.Ser.Leu.—
ro.Leu.Leu.Ser.Asp-NH_2.Thr.Asp-NH_2.Cys.Lys.—
ys.Tyr.—

is sequence of only 38 residues contains
f the basic groups in the enzyme, many
of them in an almost regular repeat, and only one acidic group. It is hard to imagine this as an α-helix!

It is difficult to avoid the conclusion that even the elementary knowledge of protein structure obtained from studies of the amino acid sequence will provide us with some surprising information that may illuminate (or obfuscate?) our picture of enzyme mechanisms.

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Comments on the Modification of Enzymes, with Special Reference to Ribonuclease

FREDERIC M. RICHARDS
Department of Biochemistry, Yale University, New Haven, Connecticut

I should like to present some comments n the enzymic and chemical modification proteins with special reference to ribo-uclease and on the bearing of the results such studies on the "active center" ypothesis.

For purposes of discussion of catalysis displacement reactions, most investi-ators find it convenient to divide the nzyme arbitrarily into a number of re-ons:

I. The small region comprising only one two functional groups in closest prox-nity to the chemical bond whose forma-on or cleavage represents the over-all nemical reaction under study.

II. Those areas, excluding I, in direct ontact with the substrate or substrates .e., regions where the interatomic dis-nces between enzyme and substrate are qual to or less than the van der Waals ollision diameters).

III. All regions more remote from the nzyme bound substrate than those in asses I and II but that still influence the nzyme activity.

IV. Those regions that can be removed altered with no noticeable effect on any spect of enzymic behavior.

The term "active site" or "active center" as been used by various people to mean gion I or regions I and II together but in l cases excluding region III. Region I has een termed the "catalytic site" and region the "binding site." When we consider the echanism of enzyme action from the oint of view of the protein chemist, the xtent to which it is sensible or realistic make such a division of the enzyme olecule is the subject of much argument.

Aside from inferences drawn from clas-cal kinetic studies, strong evidence in vor of such functional areas comes from e studies of catalysis of displacement re-actions by simple molecules. The manner in which various functional groups operating either singly or in pairs can effect catalysis is becoming clear. It seems eminently reasonable that similar groups should occur in an enzyme and perform the same functions. They would of necessity be located in region I. Intramolecular catalysis in simple compounds, such as has been discussed at this meeting, indicates the rate enhancements that may be obtained by proper steric configurations and by the change in the entropy factor normally present in bimolecular reactions. These compounds mimic at least some of the assumed functions of regions I and II. For reactions of direct biochemical interest the rates so far obtained in model systems are not of the same order of magnitude as those observed in enzyme catalysis. Model studies bearing on the influence of region III are very sparse. The fascinating observations of Wang ('58) on the effect of embedding heme groups in a nonpolar medium may be taken as an example.

The behavior of the native enzyme is clearly the result of the cumulative effect of many parts of the molecule. If region I could be excised from the enzyme with no alteration in its stereochemistry but merely removed from the influence of the rest of the molecule, I see no reason to believe that it would be a better catalyst than many of the model compounds already investigated. When region I is properly combined with a similarly separated region II, considerable improvement in catalysis would be expected but full enzymic activity would not be obtained in the absence of region III, which supplies, at the very least, the electrostatic and polar or nonpolar environment affecting I and II. Region III is frequently assumed to provide only the supporting skelton that holds

I and II in the appropriate stereochemical configuration. There is no evidence in favor of such a simplifying assumption. Indeed, this classification system has been set up purely on the basis of the spatial relations between substrate and enzyme. The existence of "linked functions" (Wyman, '48) and the possibility of energy transfer between distant parts of a protein molecule (Teale and Weber, '57; Karreman and Steele, '57) may mean that, for some enzymes at least, region III or a part of it is as important for the catalytic behavior as region I.

Unequivocal evidence for the existence of these distinct functional areas in the enzyme molecule is difficult to obtain. The experimental data to be interpreted are always of the same general nature. The enzyme molecule is altered either by exposing it to different external media (i.e., changes in *p*H, temperature, ionic strength, or solvent) or by chemical reaction in which either simple organic or enzymic reagents are used. The effect of such alterations on the activity of the enzyme is then noted. In general, there is no way of knowing whether the alteration in structure has occurred in region I, II, or III or in several simultaneously. Interpretation is thereby rendered extremely difficult. Chemical alteration of a functional group in region I might be expected to destroy all enzyme activity. A change in region II that markedly affected the binding of the substrate might easily reduce the enzymic activity by a factor of 100. Based solely on activity measurements, this situation would be experimentally indistinguishable from the first. Changes in region III that affected such factors as the net charge on the protein or the charge distribution might be expected to have much less of an all-or-none effect on the enzyme activity. However, such primary effects might easily introduce secondary changes in the stereochemistry of regions I or II and thus a very marked loss in activity that again would be indistinguishable from those already discussed. For example, interruption of an ordered helical region at a single point in the middle might result in two helical parts, which then, being separately unstable (Schellman, '55), collapse as ordered structures, although the original helix was stable. Neither region I nor might have been in the vicinity of t initial break in the helix structure.

A basic difficulty in all work on t chemical modification of proteins is o inability to specify in sufficient detail t extent of changes in the primary, secc dary, and tertiary structures produced a given set of conditions. Marked alter tions in structure are frequently reveal by accompanying changes in such pro erties as intrinsic viscosity, optical rot tion, and absorption spectra. The absen of detectable changes in such physic properties, however, is not sufficient e dence that no shifts in structure have c curred. If two amino acid residues carr ing the functional groups of region I a located on very different parts of a lon coiled peptide chain, a very small chan in the over-all physical properties of t protein may easily be accompanied enough dislocation of these residues result in complete loss of enzymic activit Reversal of a modification reaction wi concomitant recovery of activity can on imply that additional structural changes the modified enzyme, if any, are also r versible and not that such changes did n occur. From this point of view, modific tions that result in no change in enzym activity are the easiest to interpret sin it is almost certain that we are not deali with regions I or II.

With present techniques, detailed mo fication studies are practicable only wi enzymes of low molecular weight. F modification involving covalent structu a prime requirement is our ability to speci exactly the nature and extent of all t covalent changes produced. This impli evidence not only for the expected chan but also against the occurrence of a other changes (see Herriott, '54). T latter can rarely be accurately assess in proteins of molecular weight larger th 20,000 to 25,000. In general, the modi cation can be related only to the prima structure where the amino acid sequen of the protein is known. At the mome RNase is the only enzyme that fulfills these requirements.

Many modifications have been made the RNase molecule. None of these ha yet provided definitive evidence as to t

exact nature of all the structural changes produced and attempts at correlating the observed changes with the various regions mentioned have been largely unconvincing. Some examples will be discussed.

Limited digestion of RNase with the proteinase subtilisin splits peptide bond number 20, as measured from the N-terminal end of the single chain of the native enzyme (Richards, '58; Richards and Vithayathil, '59). No other change in covalent structure could be demonstrated. This modified enzyme, RNase-*S*, is uncontaminated with the native enzyme and has enzymic properties, as far as they have been measured, identical with those of the starting material in aqueous solution. These data would clearly place peptide bond number 20 and the atoms in its immediate vicinity in region IV. However, subtle structural changes have occurred. RNase-*S* can be easily digested with trypsin in aqueous solution, at least five or six bonds being split. Native RNase is completely stable to trypsin under similar conditions. RNase-S shows no enzymic activity in 8 *M* urea, again in contrast to the native enzyme (Anfinsen, '56).

The N-terminal 20 residue peptide, RNase-*S*-peptide, can be separated from the rest of the molecule, RNase-*S*-protein. Neither component shows any enzymic activity (less than 1%) by itself. The activity is largely recovered when the two components are mixed. We may now ask whether RNase-*S*-peptide or any portion of it falls in region I, II, or III of the RNase-S molecule. I can see no way of answering this on the basis of available evidence. The extremely tight binding observed (Richards and Vithayathil, '59) between the peptide and protein components can (1) impose a rigid, ordered structure on the peptide and thus confer properties not shown by the random coil; (2) cause structural changes in the protein component resulting in the appearance of catalytic activity; or (3) produce sites I and II or both in the contact region between the two components. In this last case, the binding of the substrate would certainly be expected to influence the binding of the peptide to the protein. Such an effect has been inferred from studies on a modified peptide (Richards and Vithayathil, '59). However, any of the three possibilities described would lead to these observed results if the substrate binds more tightly to the active enzyme than it does to RNase-*S*-protein. (The nature of the forces responsible for binding the peptide are obviously of interest for the basic problem of protein secondary structure but they will not be considered further here.)

The imidazole group of histidine has been repeatedly implicated as at least one member of region I in a number of enzymes. The situation in RNase is anything but clear. Weil and Seibles ('55) subjected RNase to photooxidation in the presence of methylene blue. During the earlier stages of the reaction most of the enzymic activity disappeared, histidine was the only amino acid affected, and the relative viscosity and optical rotation of the solution were unchanged. These authors concluded that histidine residues were important for the activity of the enzyme but were careful to draw no conclusion as to whether one or more residues were involved or the manner in which they exerted their effect. RNase-*S*-peptide contains one of the four histidine residues present in the native enzyme. When this peptide is subjected to photooxidation under conditions similar to those used by Weil and Seibles, it loses all ability to regenerate enzyme activity when remixed with RNase-*S*-protein (Richards, '58). RNase-*S*-protein, containing the other three histidine residues, is also inactivated by photooxidation. The implication of these findings is that not less than two histidine residues must be intact in the active enzyme.

The original observation of Zittle ('46) that iodoacetate inactivates RNase has been reinvestigated in a number of laboratories. Stein and Barnard ('58) reported on the inactivation by bromoacetate, identified carboxymethylhistidine in the inactive protein, and located the site of reaction as the histidine residue nearest the C-terminal end of the peptide chain of RNase. Grundlach *et al.* ('59) reported on the inactivation by iodoacetate. The enzyme loses activity faster at *p*H 5–6 than at 4 or 8. The rate of inactivation increases again below *p*H 4. This maximum in the rate of inactivation has also been observed by B. Weinberg and Richards (unpublished

results). Grundlach *et al.*, ('59) further found that at *p*H 6 the only amino acid residue affected by the reaction was histidine, and at *p*H 8 histidine did not react but lysine did. At *p*H 3 neither of these reactions occurred, but the sulfonium salt of methionine was formed. These odd results would not be expected on the basis of the reaction of simple models with iodoacetate. The reaction with the sulfur of methionine would be expected to be *p*H independent. The imidazole and amino groups would be expected to react only in the basic form. The altered reactivity of these functional groups in the protein must be caused by structural factors as yet unknown. It is important that these experiments be repeated with iodoacetamide to determine the extent to which electrostatic repulsion of the iodoacetate ion is involved. RNase-*S* and RNase-*S*-protein are inactivated by iodoacetate at *p*H 6, whereas RNase-*S*-peptide loses activity more slowly. The apparent discrepancy between the results of Stein and Barnard ('58) and these studies on RNase-*S* and its components has not yet been resolved. The only conclusion to be drawn at this time is that there are probably at least two histidine residues in RNase, the reaction of either one of which results in the loss of enzymic activity. No conclusions are warranted as to whether these residues are in region I, II, or III.

Many chemical reagents known to react with amino groups inactivate RNase. Formaldehyde, ninhydrin, and phenylisocyanate were used by Zittle ('48), *O*-methylisourea by Klee and Richards ('57), Geschwind and Li ('57), and Brown *et al.* ('57), diphosphoimidazole by Taborsky ('58), and, as mentioned, iodoacetate at alkaline *p*H by Grundlach *et al.* ('59). All these studies suffer from the general difficulties of chemical modification discussed. Modification of RNase-*S*-peptide is in a different category. There is no evidence that the peptide has any rigid secondary structure when freed from the protein. It cannot be irreversibly denatured in the usual sense. Because of its small size, covalent changes in its structure can be established with reasonable certainty. If enzymic activity is altered when a modified peptide is mixed with RNase-*S*-protein, the initiating factor responsible for this change can with certainty be said to b the known change in the covalent struc ture of the peptide. By the reasoning a ready given, however, it is not possible o the basis of these data alone to place th altered portion of the peptide in region II, or III of the reconstituted enzyme.

Three of the amino groups present i native RNase are present in RNase-S peptide, whose sequence is known fro the work of Hirs and associates ('58 and is given:

Lys.Glu.Thr.Ala.Ala.Ala.Lys.Phe.Glu.Arg.Ser.
Thr.Ser.Ser.Asp.His.Met.Glu.Ala.Ala

The histidine residue, whose modificatio was discussed, is near the C-terminal en of the peptide. The two lysine residue near the N-terminal end provide the thre amino groups, one α and two ε. Thre derivatives were prepared (P. Vithayathi unpublished results): *Deam-S-peptide*— Treatment of *S*-peptide with nitrous aci converted the α-amino group to a hydroxy group. The two ε-amino groups and a other residues were unchanged. (Tenta tive structure assignment is on the basi of available evidence.) *Guan-S-peptide*— Treatment of *S*-peptide with *O*-methyliso urea converted the two ε-amino groups t guanidino groups. The α-amino group an all other residues were unchanged. *Acety S-peptide*—Treatment of *S*-peptide wit acetic anhydride resulted in the acetyla tion of all three amino groups. All othe amino acid residues were unchanged.

When a slight molar excess of Guan *S*-peptide is mixed with RNase-*S*-protei about 80% of the activity to be expecte for the unmodified peptide is regaine whether RNA or uridine-2′:3′-phosphat (Ur!) is used as a substrate. When RN is used, this activity ratio is maintained a all *p*H values tested between 4 and 1 When Deam-*S*-peptide is used, the sam statements are roughly true, except tha the activity regained is somewhat highe and may be slightly different for the tw substrates. The data of Tanford an Hauenstein ('56) on the ionization RNase do not indicate any abnormalitie in the ionization of the amino groups. appears then that the ionization of th three amino groups of RNase-*S*-peptide ar not related to the activity of the enzym

since their ionization behavior can be markedly altered without effect on the *p*H-activity profile. The results on Guan-*S*-peptide confirm the earlier work of Klee ('58), who concluded that the activity loss on complete guanidination of native RNase could not be attributed to the changes in the two lysine residues nearest the N-terminal end of the molecule. RNase-*S*-protein loses activity on guanidination in much the same manner as native RNase.

With acetyl-*S*-peptide the same experiments give only about 35% activity with RNA, and this value is unchanged even with a large molar excess of the peptide. When the low molecular weight substrate, Ur!, is used, the activity does not reach a maximum with molar ratios of peptide to protein close to 1, but gradually increases with higher ratios and approaches the activity to be expected for the unmodified peptide. Using RNA as substrate, the relative activities (35–40%) of acetyl-*S*-peptide and the unmodified peptide are the same at all *p*H values.

Since an alteration of charge at the three amino groups does not seem to be reflected in an activity change and since, sterically, guanidino and acetyl groups are very similar in size, it would appear that the RNA activity loss with acetyl-*S*-peptide is principally caused by a steric effect of the acetyl group in the α-amino position. This hypothesis is arrived at by elimination and therefore stands on very shaky ground. Further chemical and enzymic modifications now under study may clarify the situation. The amino groups of RNase-*S*-peptide are certainly not in region I or IV and are probably in III when Ur! is the substrate and II when RNA is the substrate.

The examples of modification studies given are sufficient to indicate the difficulties attending present attempts to identify the functional significance of the various portions of an enzyme molecule. The question may be asked whether these studies on RNase have any bearing on the conclusions to be drawn concerning other enzymes (esterases, trypsin, chymotrypsin) whose "active sites" have been studied by chemical modification with reagents such as diisopropylfluorophosphate.

OPEN DISCUSSION

KALNITSKY[1]: Dr. Carter, at Iowa, has developed a method for cleaving disulfide bonds. It is essentially a sulfitolysis method, which works well with all the proteins that we have tried, and we have adapted it to cleaving the disulfide bonds of ribonuclease.

With one of the four disulfide bonds cleaved, in a number of different experiments, 88–100% activity remained and it did not make much difference whether we used a native RNase or RNase "A" separated on an Amberlite IRC-50 column. With two disulfide bonds split, we invariably found that about 80% of the activity remained. This confirms Dr. Anfinsen's results, which were obtained with a different method. But the interesting thing to us is that when we split 3.9 of the 4 disulfide bonds in *6 M urea*, in about seven or eight experiments carried out in duplicate (and the duplicates run in duplicate) about one-third of the activity always remained. However, when we got total cleavage of the disulfide bonds in *8 M urea*, complete inactivation occurred. This would indicate that after the splitting of the disulfide bonds, a rearrangement or alteration in the structurally weakened molecule takes place that results in denaturation and inactivation of the enzyme. This would imply that the secondary structure is necessary for the activity of this enzyme.

We performed some additional experiments that show this more clearly. Harrington and Schellman at the Carlsberg Laboratory and Weber and Tanford at Iowa, using viscosity and optical rotation measurements, showed that the RNase molecule starts to unfold between 50° and 58°C. We determined the initial velocities of this enzyme in the folded and unfolded state from 30°–80°C., at 5° intervals. The ratio of the initial velocity at 40°C. to that at 30° is 2.77, representing almost a threefold increase with a 10° rise in temperature. About the same increase is obtained going from 40° to 50°C., but between 50° and 55° there is a drop in the Q_{10} value.

[1] George Kalnitsky, State University of Iowa.

By plotting the log of the initial velocity against 1/T on the Kelvin scale, we get the usual Arrhenius plot. The points obtained between 30° and 50°C. fall on a straight line; the points obtained at 55° and above do not fall on this line. This drop in activity occurs just where the molecule starts to unfold and, according to Sizer, represents a shift in the configuration of the protein molecule. Interpreting these experiments on the heat denaturation of RNase in aqueous solution in terms of a single reaction (i.e., native protein ⇌ denatured protein) as did Harrington and Tanford, we obtained values, for this reaction, of ~ 37 kcal/mole and 110 entropy units, respectively, for $\Delta H°$ and $\Delta S°$. These values are reproducible and are quite a bit lower than those obtained by Harrington and Schellman and by Weber and Tanford. They were obtained with three different enzyme concentrations and with three different RNA preparations. Our results would indicate that at least some portion of the secondary structure seems to be essential for the catalytic activity of the enzyme molecule and that a limited rearrangement or disorganization of the molecule leads to denaturation and inactivation.

WEIL[2]: I do not know whether I understood you well. Did the two fractions of RNase that resulted from the hydrolysis both react with iodoacetic acid and combine with histidine?

RICHARDS: We do not know exactly what happens during the reaction, but the separated fractions are both inactivated by treatment with iodoacetate.

WEIL: You did not isolate the substituted histidine?

RICHARDS: No, we have not analyzed for it yet.

COHEN[3]: I would like to suggest another possible approach to the question of active enzymic sites. Drs. M. Marshall and R. L. Metzenberg in my laboratory have prepared a highly purified enzyme, carbamylphosphate synthetase, and have also produced a highly specific antibody to it. The remarkable feature of this antibody is that it seems to be specific for the constituent groups required for enzymic activity. Thus, at the equivalence zone, the enzymic activity is completely blocked. Furthermore, the antibody reacts with extracts of liver from *other species* known to contain this enzyme (ureotelic animals) but not at all with extracts of liver from nonureotelic animals. The possibility is thus suggested that degradation of this enzyme to suitable polypeptide fragments would yield a fragment containing the amino acid sequence capable of blocking or competing with the antigen for the antibody. This approach, if successful, would provide some indication as to the amino acid sequence involved in the enzymic activity. I am not aware that this approach has been used to aid in the characterization of an enzymic site. I have not pursued this beyond that point.

HANDLER[4]: Is it generally true that such antibodies do not show any species specificity?

COHEN: I do not believe that this is generally true.

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[2] Leopold Weil, Eastern Regional Research Laboratory of the U.S. Department of Agriculture.

[3] P. P. Cohen, University of Wisconsin.

[4] Philip Handler, Duke University.

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Some Approaches to the Study of Active Centers

CHRISTIAN B. ANFINSEN[1]
Laboratory of Cellular Physiology and Metabolism, National Heart Institute, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland

Although we are far from an understanding of the actual molecular basis of ribonuclease (RNase) action, a number of studies have been carried out that indicate the essentiality or nonessentiality of various parts of the sequential structure and of the secondary and tertiary structures of this enzyme. These studies have been discussed a great deal in the past and a detailed repetition would be superfluous (Anfinsen, '58; Moore and Stein, '56–'57; Richards, '58). Richards (this Symposium) summarized many of the high points, and Kalnitsky reviewed some of the correlations between activity and secondary structure. For these reasons, I should like only to include the schematic and rather speculative drawing shown in figure 1, which sums up many of the observations for which there is reasonably good experimental support.

The observations summarized in this figure, together with the material presented by Richards and Kalnitsky make it possible to state with some assurance that not all of the structure of RNase is essential for activity *as measured in in vitro test systems*. A wealth of similar information on other proteins and polypeptides indicates that this conclusion is probably a general one (Anfinsen and Redfield, '56; Smith *et al.*, '58; Li, '56, '57). In the context of the evolutionary process we must accept the probability that the process of natural selection would never permit the perpetuation of "nonessential parts" unless they furnish some very real selective advantage to the organism in question. The true importance of those portions of a biologically active molecule that seem to be unessential *in vitro* can be appreciated only when we learn how to study catalytic activities in natural, intracellular environments where the properties of enzymes may be expected to be both species and tissue specific.

Species variation. It might be worth while to outline the general hypothesis (Anfinsen, '59) that underlies a good deal of our own approach to the active center question. We assume that the changes that take place in the structure of a particular protein during speciation are the direct or indirect result of gene mutation and that natural selection furnishes the screening procedure. It is further assumed that only those mutations that result in a beneficial, or at least nonharmful, change will survive this screening process and that mutations leading to a change in the more "carefully" designed catalytic center would be lethal. Thus, whereas the catalytically active portion of an enzyme might be extremely similar whatever the organism of origin, the peripheral, species specific parts of the molecule might undergo considerable structural change in the course of evolution in conjunction with similar changes in the other protein components of cells with which it must harmoniously interact. Although not an obligatory part of this hypothesis, it is most tempting to suggest that certain parts of the substructure of genes [or cistrons (Benzer, '57) to be more accurate] in presently living organisms are *direct* descendants of similar parts of genes in organisms long since extinct and that there has been a real continuity in the evolution of the gene pool. Convergent evolution with the independent rediscovery of the same genetic solution to a functional problem would also account

[1] Illness in Dr. Anfinsen's family prevented his attendance at the Symposium, but this paper represents a comprehensive summary of the talk he had prepared.

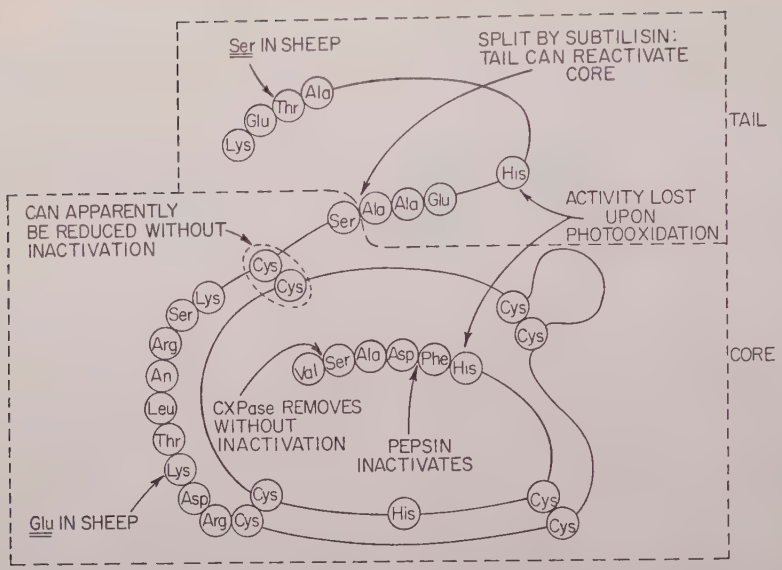

Fig. 1 A schematic summary of some of the observations that have been made relating structural features of bovine pancreatic RNase to its biological activity. The figure suggests that certain specific points in the structure, such as the two histidine residues and the peptide bond between residues 120 and 121, are apparently required for normal function. Other parts of the structure, such as the residues that are changed in the enzyme isolated from sheep pancreas, the disulfide bridge between half-cystines no. 1 and no. 6, and the C-terminal valine residue, seem to be less critical features.

for structural similarities, but seems clumsier and less efficient as a working hypothesis.

The provocative experiments of Tuppy and his colleagues on the structures of the heme-peptide of various cytochrome c molecules from different organisms (Tuppy, '58) and of Sanger ('59) on the insulins already show how similar such structures can be in widely different organisms. At the other extreme, a number of proteins that differ quite widely in different organisms have been studied. The serum proteins, for example, show complete lack of immunologic cross-reactivity when fairly distant species are compared (Nuttall, '04). This sort of evidence, although not necessarily a good indication of structural difference, does suggest that some proteins may be modified extensively without serious impairment of biological function and, indeed, we now know that certain individuals can lead more or less normal lives in the *complete* absence of serum albumin. One gets the general impression that the degree to which a particular protein can be modified during evolution is a function of the exactness of its molecular desig with respect to functional needs, and tha individual proteins may be assigned a pos tion in a spectrum of "violability" rangin from precise structural dependence of fun tion to very slight dependence for the mo nonspecific functions, such as amino ac storage, osmotic pressure regulation, ar the transport of smaller molecules.

The "common denominator" approac The most general method for studying th structural requirements for activity in specific enzyme involves the systemati well-controlled degradation of the enzym the isolation and purification of derivative and the simultaneous examination of th effects of the degradative procedures function. On the other hand, the "comm denominator" approach through speci comparison requires techniques for th comparison of the complete structure ideally by complete sequential analysis alternatively by methods that give a go qualitative idea of sequence through th isolation of peptide fragments prepared a uniform way. The highly developed c umn chromatographic methods and t

"fingerprinting" techniques that involve successive chromatography and electrophoresis on paper now make the latter sort of characterization feasible for most proteins of reasonable size. I should like to present two examples of how species comparisons might be valuable in locating the active centers of enzymes, one having to do with RNase and one with lysozyme.

Taking advantage of the fact that we now have an essentially complete covalent structure for bovine pancreatic RNase, we have subjected this protein and the pancreatic RNases of the sheep (Åqvist and Anfinsen, '59; Anfinsen *et al.*, '59) and pig to the fingerprinting technique. This technique, which has been used so elegantly by Ingram in his studies on the differences between normal and abnormal hemoglobins (Ingram, '57), has been somewhat modified to give sharper and more reproducible peptide patterns (A. Katz, W. J. Dreyer, and C. B. Anfinsen, submitted for publication). For the present purposes, it is sufficient to state that the proteins were either oxidized with performic acid or subjected to reduction and alkylation for the purpose of converting the native molecule to an extended, more easily digested form. The polypeptide chain was then digested, either with trypsin alone or with successive treatment with trypsin and chymotrypsin. After separation of the peptides on paper sheets by chromatography and electrophoresis, each peptide was eluted and analyzed, qualitatively, for amino acid composition. The bovine and porcine enzymes gave identical fingerprint patterns (fig. 2) and, indeed, upon elution and analysis of the individual components, absolutely no differences in composition could be detected, although of course the possibility of inversions in small portions of the sequence cannot be ruled out without a thorough sequential analysis of each peptide. The sheep enzyme, on the other hand, differs significantly from bovine RNase, and two of the differences are shown in detail in figure 3. The sheep enzyme contains serine in place of a threonine residue and a glutamic acid instead of a lysine residue. There is good evidence for the presence of a third difference between these two proteins involving the replacement of asparagine by glutamine, although this

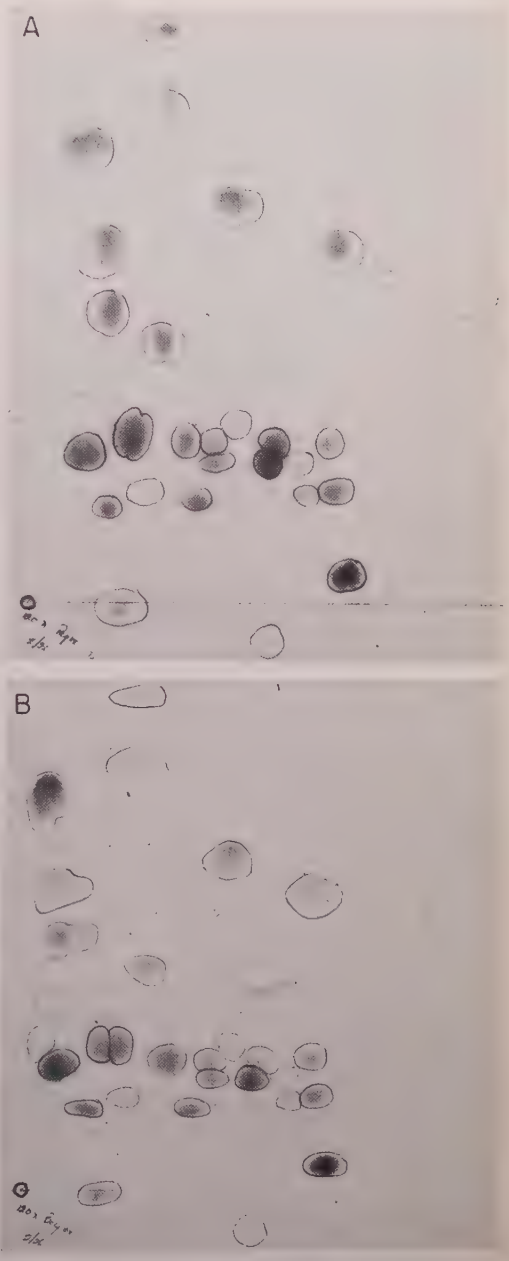

Fig. 2 "Fingerprints" of trypsin-chymotrypsin digests of oxidized porcine (A) and bovine (B) pancreatic RNases. All the peptide components of the porcine enzyme, on elution and analysis, were identical with the corresponding peptides in the digest of the bovine enzyme, within the experimental error of the paper chromatographic methods used.

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The Aminoacyl Insertion Reaction

M. BRENNER
University of Basel, Basel, Switzerland

The purpose of this paper is to draw attention to some chemical discoveries that are likely to influence present ideas on peptides. In particular, a novel kind of rearrangement will be discussed. This reaction involves an aminoacyl residue; for example, the residue of an α-amino acid, which during the process is inserted between the carbonyl and the imido group of an amide bond. These characteristic features have led us to propose the term "aminoacyl insertion reaction" as a name for this type of chemical change (Brenner, '58a).

Emil Fischer and his school looked upon a peptide as being in some ways analogous to a string of pearls. The analogy must have been felt quite strongly. Until now, this situation has been reflected in all approaches to peptide synthesis and degradation and in a firm belief in sequence stability. Synthesis, for example, has been exclusively considered in terms of end-to-end addition of chain components. Degradation has always been seen as a cutting process, and intramolecular sequence changes in a completed peptide chain have been thought impossible.

These views are not broad enough. Peptides do have properties that fit in with the analogy of the string of pearls, but they possess others that clearly are at variance with it.

The synthesis of a peptide chain will be considered first. The left side of figure 1 schematically illustrates the classical approach. The chain is elongated by the addition of one reactant to either end of the second reactant. The right side of figure 1 illustrates a new alternative for chain elongation, this time by insertion. This cannot be done in a string of pearls, but an amino acid can be inserted into a peptide. In principle, two conditions must be fulfilled to allow insertion to occur: (1) A carboxyl-activated amino acid must be brought close to a peptide chain, and (2) a suitable proton acceptor must be provided to initiate the reaction.

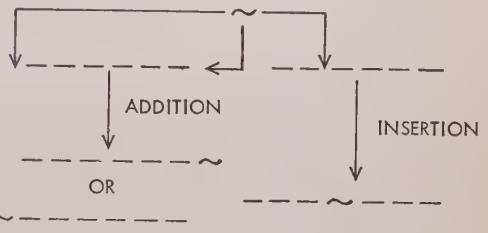

Fig. 1 Schematic illustration of addition and insertion in the construction of a peptide chain. The symbol ~ indicates an amino acid residue to be added or inserted, and the symbols — — — — — —, — — — — — — — ~, ~ — — — — — — —, and — — — ~ — — — indicate peptide chains.

I shall return to this general case but first it is necessary to discuss some specific results. If, for example, the *O*-peptides in table 1 (left side) are subjected to the action of a strong base, such as *t*-butylate ion or amide ion, they rearrange in a fast reaction with good yields into the products shown. It will be noticed that, contrary to what occurs in the *O,N*-acyl migration (Bergmann and Miekeley, '24; Elliott, '52; Josefsson, '58), the amino group of the hydroxyamino acid or mercaptoamino acid does not participate in the insertion reaction. It must, however, be protected by an acyl group.

The products shown in table 1 are alcohols and can obviously be esterified with further amino acid molecules. Rearrangement of these esters would then produce new peptide derivatives, which could again form esters, and so on. Several successive insertion steps of this type were performed with certain derivatives of salicylic acid. Thus, starting from *O*-glycylsalicylic acid (I), the peptide derivative salicoyl—glycyl—phenylalanyl—glycine methylester

TABLE 1

Demonstration of the aminoacyl insertion reaction with peptide models containing serine, threonine, allothreonine, and cysteine

All products except those marked with an asterisk were isolated in the crystalline state in good yield. The configurations of the serine, threonine, and *allo*threonine were preserved (*K*—*t*-butylate, *t*-butanol).

H.Gly.O | Bzo.Ser.NH_2	⟶	Bzo.Ser.Gly.NH_2
H.Gly.O | Bzo.Gly.Ser.NH_2	⟶	Bzo.Gly.Ser.Gly.NH_2
H.Phe.O | Bzo.Ser.Gly.NH_2	⟶	Bzo.Ser.Phe.Gly.NH_2*
H.Gly.O | Bzo.Thr.NH_2	⟶	Bzo.Thr.Gly.NH_2
H.Gly.O | Bzo.*allo*Thr.NH_2	⟶	Bzo.*allo*Thr.Gly.NH_2
H.Gly.S | Bzo.Cy.NH_2	⟶	Bzo.CySH.Gly.NH_2*

Bzo = C_6H_5CO.
* Identified by paper chromatography.

(III) was prepared (Brenner *et al.*, '55; Brenner, . . ., and Hartmann, '57; Brenner, . . ., Beglinger, '57; Brenner and Zimmermann, '57, '58; Brenner and Wehrmüller, '57) as illustrated by I–III in figure 2. It will be noticed that rearrangement in the aromatic series requires less-basic conditions than in the aliphatic series. This is because the rigid benzene ring holds the reactive centers in a sterically favorable position. Except for that effect, there is no difference between insertions in the two series. Special attention should be given to the rearrangement of *O*-glycylsalicylic acid (I) into salicoylglycine because it occurs with participation of a free carboxyl group. An equivalent reaction with a derivative of an aliphatic hydroxy acid has not been demonstrated. There is, however, good reason to assume that, under more favorable reaction conditions, insertion into an aliphatic carboxyl group can also occur.

The materials used in all these experiments have low molecular weight and are not free peptides. They constitute, never-

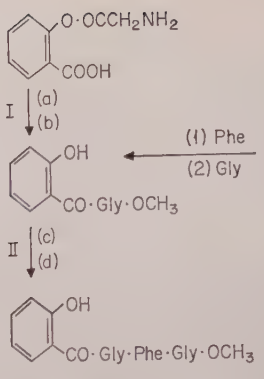

Fig. 2 (a), Rearrangement in water; (b), es terification with CH_2N_2; (c), rearrangement afte reaction (1); (d), rearrangement after reactio (2) (chloroform triethylamine).

theless, very satisfactory peptide models For our present purpose, the differenc between the models and true peptides i not important. The essential conclusion to be drawn from the results so far pre sented may be summarized as follows:

(1) The amino acid to be inserted i activated by esterification. [The process o esterifying amino acids was shown in earl work (Brenner *et al.*, '50) to result i activated, energy-rich derivatives.]

(2) The esterified amino acid is force into close proximity with a peptide bond Such close proximity is secured by virtue o the esterifying hydroxyl or mercapto grou itself being an integral part of the peptid structure into which the activated amin acid is to be inserted. Such proximity in creases the probability of collision betwee the activated amino acid and the peptid chain.

(3) The insertion is base catalyzed.

Details have yet to be worked out befor insertion by way of hydroxy or mercapt acids will be generally useful for the chem ical synthesis of peptides. This type o insertion reaction, however, illustrates possible mechanism for peptide synthesi in biological systems. Figure 3 shows wha may occur in biological systems. The cas shown in this figure is chemically mor specific than that shown on the righ side of figure 1.

In further work on the aminoacyl inse tion reaction, we discovered that the r action was more general than would b

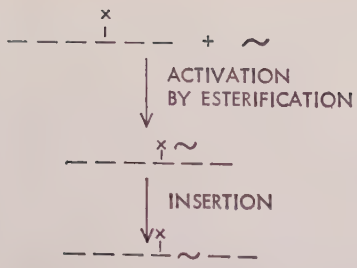

Fig. 3 Schematic illustration of insertion by ray of β-hydroxy- or β-mercaptoamino acids. he symbol X indicates a residue of serine, hreonine, or cysteine. For other symbols, see gure 1.

ndicated by the seryl- and salicoylamino cid ester reactions. *O*-Glycyl-β-hydroxy-utyric acid amide (IV, fig. 4) corresponds n structure to the threonyl derivative in able 1 and rearranges in the same way, s was to be expected. It was, however, urprising to see that variations in chain ength of both the amino and the hydroxy acid parts of the molecule (see V, VI, and VII, fig. 4; Brenner and Quitt, '57) did not seriously affect the capacity for undergoing an insertion reaction. There is evidently some choice in the means of connecting reactive centers, which consist of the amide bond, the ester bond, and the terminal amino group in each of the materials IV, V, VI, and VII. Provided that these reactive centers are brought together, either spontaneously or guided by chemical attachment or a catalyst surface, the number of intervening atoms forming the link is not significant. These may not all be carbon atoms and they may constitute a heteroatom chain, e.g., a peptide chain. It is conceivable from such an extrapolation that a serine hydroxyl in a polypeptide could, in a first step, esterify an α-amino acid or a peptide and insert these in a second step at any peptide bond on the carboxyl side of the serine residue. Finally, the ester bond and the peptide bond par-

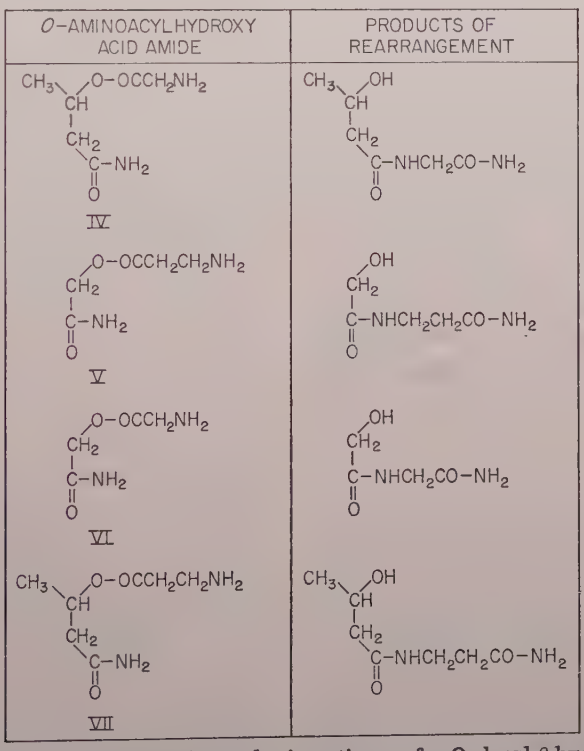

Fig. 4 Rearrangement by aminoacyl insertion of *O*-glycyl-β-hydroxybutyric acid amide, *O*-(β-alanyl)-glycolic acid amide, *O*-glycylglycolic acid amide, and *O*-(β-alanyl)-β-hydroxybutyric acid amide. All products were isolated in the crystalline state and identified by comparison with authentic specimens.

ticipating in an insertion reaction might not be linked together. Under these circumstances the general scheme shown on the right side of figure 1 would become feasible. So much for the synthesis.

Degradation by reverse aminoacyl insertion has not yet been observed. With the substances in the right column of table 1 as starting materials, this degradation would involve, in a first stage, ester formation with concomitant disappearance of an amide bond and, in a second stage, splitting of the ester bond. The first reaction step requires a considerable amount of energy and, correspondingly, has a very small equilibrium constant. Under the experimental conditions so far investigated, the over-all reaction may therefore be slow compared with the decomposition of the molecule by other routes. The reverse of the aminoacyl insertion reaction, however, seems possible if coupled with an energy-yielding process.

Possible variations and extensions of the aminoacyl insertion reaction are even greater than has so far been indicated. As a consequence, the concept of sequence stability in a peptide is not necessarily valid. In the examples given, the aminoacyl residue to be inserted was activated by esterification. Other types of activation are known in peptide chemistry and should be applicable to the aminoacyl insertion reaction. In enzymic peptide synthesis by transamidation, for example, amidation instead of esterification was used for activation (Fruton, '56). In the aminoacyl insertion reaction, amidation should likewise be an alternative method to esterification for activating the amino acid to be inserted.

Replacing the hydroxyl oxygen in the formula of *O*-(glycyl)-glycolic acid amide (VI, fig. 4) by —NH, gives the structure of the dipeptide amide, glycylglycinamide. If the aminoacyl insertion mechanism operates in this peptide in the same manner as in the *O*-peptide (VI), the effect must be a reversal of the sequence of glycine residues. The analogy between the two cases is complete, except for the driving force. This probably amounts to several thousand calories in the ester rearrangement (Brenner, . . ., and Beglinger, '57), but is small in the peptide rearrangement, the free-energy change being zero if the dipeptide ami is composed of two identical amino acid Whereas *O*-peptide → *N*-peptide conve sions go to completion (table 1), *N*-peptic conversion can yield only equilibrium mi tures. In an experiment with glycylglyci amide the detection of the rearrangeme would require some sort of labeling ar some degradation work. The analytic problem becomes simpler when a dipeptic amide of the general structure A—B—NH is used, the expected result of the r arrangement being a mixture of tw amides, A—B—NH_2 and B—A—NH which can be separated from each othe Experiments were done, therefore, wit glycylphenylalaninamide and phenylal nylglycinamide. The effect of 0.1 *M* butylate ion on each of these materia was exactly as expected: standing at roo temperature for 30 minutes was sufficie to effect rearrangement of each amide what appears to be an equilibrium mixtu of the two amides. Amide ion in liqui ammonia exerts the same effect. In th case, however, the equilibrium is ve much on the side of phenylalanylglyci amide. This material was obtained in pure crystalline state by rearrangement the isomeric amide, followed by separatic from the equilibrium mixture by ion-e change chromatography. A corroboratir result was obtained with glycylleucinamic and leucylglycinamide.

The latter findings are intriguing b cause there is no theoretical reason th should limit isomerization to dipeptic amides. The extrapolations from the peptide rearrangements apply in an ana ogous manner to peptide isomerizatio Thus we arrive at a concept, "sequen tautomerism," which is far-reaching in i implications. Isomerization of a peptide a specific sequence A.B.C. . . . X.Y.Z any peptide of composition [A,B,C, . . . X,Y,Z] by a number of successive insertic steps would appear to be merely a que tion of reaction rates or, in other word of the presence of appropriate catalyst During the whole process the size of th chain would remain unchanged.

It is important to note that, for a possib biological role, the aminoacyl insertion r action would have to occur in the presen of water. Unlike rearrangements in th

alicylic acid series, however, insertion in he aliphatic series seemed to require an- ydrous reaction media. Compared with he rates of hydrolytic reactions, insertion vas thought to be too slow to be detected nalytically. A closer examination of the roblem revealed that this is not so. Both)-peptide rearrangement and peptide iso- nerization occur as detectable reactions in dilute sodium hydroxide solution (0.1–0.25 N), although hydrolyses of the ester groups nd sometimes of the amide groups may ecome the principal reactions. Dilute queous solutions of calcium hydroxide, rginine, and especially guanidine have imilar effects. Insertion also takes place in he presence of protamine solutions (clu- eine, salmine, iridine) previously treated vith excess Amberlite IRA-400 (OH^- orm). For the reactions in water, how- ver, the best results were obtained with he strongly basic Amberlite IRA-400 (OH^- orm) directly. With esters the ratio of nsertion to hydrolysis was about unity. The time required was less than 15 minutes t temperatures of 0°–25°C., and the ratio f resin to substrate in terms of titration quivalents was about 3:1. With the di- eptide amide under the same conditions, stablishment of the rearrangement equi- ibrium was considerably faster than the ydrolytic side reactions. Our impression rom such findings is that the catalytic ffect of a basic surface is more pro- nounced than the catalytic effect of a basic olution. This is not surprising since the eaction is clearly dependent on steric actors.

Rearrangements in water as the solvent ave not yet been worked out to a stage where they could be of preparative value. These observations, however, indicate that he aminoacyl insertion reaction might take lace in living cells if appropriate catalysts re present.

A clear picture of the potential signifi- ance of aminoacyl insertion in peptide nd protein chemistry is gained from the chematic summary of present knowledge fig. 5A,B) and extrapolations (fig. 5C,D) nade therefrom.

These extrapolations might be construed o mean that the structure of peptide hains is essentially very labile. The ques- ion of why peptides can retain specific sequences then arises. Either aminoacyl insertion is not a generally valid reaction possibility or there must be some device

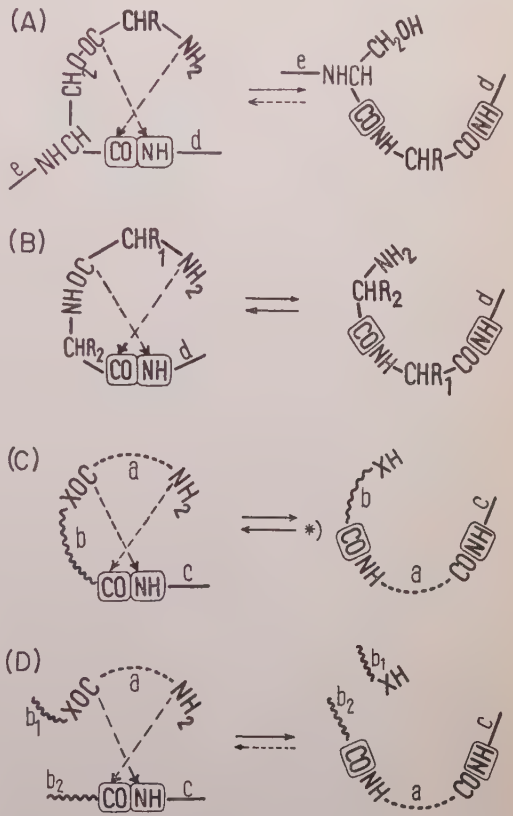

Fig. 5 Generalization of the aminoacyl insertion reaction by extrapolation from special cases. A: *Special case 1*—*O*-aminoacyl-seryl peptide rearrangement. B: *Special case 2*—peptide isomerization. C: *First extrapolation*—general intramolecular aminoacyl insertion reaction. $CONH\overset{c}{—}$ may be replaced by COOH (X=O, S, or NH) (*if X=O or S, this arrow applies only in a restricted sense—see text). D: *Second extrapolation*—intermolecular aminoacyl insertion reaction. *a* and *b* are homo- or heteroatom chains with or without side groups and are at least one carbon long; *c* is hydrogen or a homo- or heteroatom chain; *d* is hydrogen or a continuing peptide chain; *e* is a continuing peptide chain. The reaction is still theoretically feasible if *b* is of great length. In fact, there is no difference in that case whether the two parts of the chain *b* (b_1 and b_2) are actually joined. If they are not joined, then the reaction becomes intermolecular. If chain *b* is long or broken, some outside factors will have to come into play to define the point of insertion of an activated amino acid or peptide into a peptide chain; X=O, S, or NH.

preventing or slowing down spontaneous changes. Such devices would be particularly important in biological systems. There is indeed evidence for two or possibly three factors of this kind. We became aware of the first when certain expected insertions failed to take place. There was no *O*-peptide rearrangement of *O*-glycyl-*N*-benzoylserine methylamide (VIII) or isomerization of H.Leu.Gly.Gly.NH_2 (IX), H.Gly.Leu.Gly.NH_2 (X), or H.Gly.Gly.Leu.NH_2 (XI). VIII is an analog of *O*-phenylalanyl-*N*-benzoylserylglycinamide (table 1); IX, X, and XI can be regarded as *N*-substituted dipeptide amides and are therefore analogous to the amides of Phe.Gly, Gly.Phe, Gly.Leu, and Leu.Gly. Because of their analogy to amides that can undergo the aminoacyl insertion reaction, VIII, IX, X, and XI were all expected to rearrange in the usual manner. Their failure to do so demands an explanation. It is very possible, from studies on the mechanism of the rearrangement, that the first step of the reaction is loss of a proton from the peptide bond at which insertion will take place. For the expected reaction of VIII, the group in question should be the methylamide group, and in IX, X, and XI it should be the central peptide bond. Because of the suppression of anion formation by a neighboring negative charge, ionization at a required position may be prevented if other groups ionize first. This is perhaps what happens in the examples under consideration. It seems probable that the proton leaves the amide nitrogen of the —CO—NH—methyl group of VIII more slowly than does the corresponding proton of the —CONH—methylene—carboxamide portion of *O*-phenylalanyl-*N*-benzoylserylglycinamide, and still more slowly than the proton from $CONH_2$ of *O*-glycyl-*N*-benzoylserinamide. Thus the first proton lost from VIII presumably comes from the benzamide part of the molecule. In IX, X, and XI, on the other hand, primary ionization might take place at the —$CONH_2$ group. The validity of these explanations will have to be explored in appropriate experiments. In any event, it is reasonably safe to assume that ionization, including ionization of acidic groups in amino acid side chains, plays a powerful role in preventing random ionization on the peptide backbone ar random insertion reactions.

If ionization in IX, X, and XI actual takes place at the terminal —$CONH_2$ grou then insertion of either the N-termin amino acid residue or of the N-termin dipeptide residue would be expected give products of the following kind:

IX → H.Gly.Gly.Leu.NH_2 or H.Gly.Leu.Gly.NH
X → H.Leu.Gly.Gly.NH_2 or H.Gly.Gly.Leu.NH
XI → H.Gly.Leu.Gly.NH_2 or H.Leu.Gly.Gly.NH

Actually, no change of this kind was d tectable. The reason for this may be ster and may indeed be similar to the reaso that preclude the formation of cyclic t peptides in cyclization reactions (Schw zer, '58). In view of Schwyzer's statemer and of our suggested mechanism of th insertion reaction (see below), this resu could have been predicted. The second fa tor acting against random insertion ma thus be a kinetic control in that certain r quired rearrangement intermediates for so slowly that other reactions become pr dominant.

A third factor mitigating against rando insertion may be seen in thermodynam stabilities. In this context it is interestin to note that the equilibrium between glycy phenylalaninamide (XII) and phenylal nylglycinamide (XIII), which tends favor XIII, is much influenced by the so vent. The amount of XII in an equilibriu mixture is about 40% in *t*-butanol, 10% liquid ammonia, and almost zero in wate A study of thermodynamic sequence stabi ities in larger peptides would seem to b particularly interesting because of amin acid sequences in natural peptides and pr teins. It must await development or th discovery of a suitable catalyst.

In connection with the catalytic contr of the insertion reaction, attention shou be focused on its mechanism. Work don in this direction, which is discussed els where (Brenner, '58b), leads to a tentativ formulation that, for the rearrangemer of *O*-glycyl-*N*-benzoylserinamide, is repr sented by XIV–XX (see fig. 6). The rat determining step is considered to be form tion of the cyclic anion (XVI). Its co version to the bicyclic anion (XVII) hypothetical. Another route would seem be formation from XVI of a diacylimid (benzoylseryl)glycylimide, and its intr

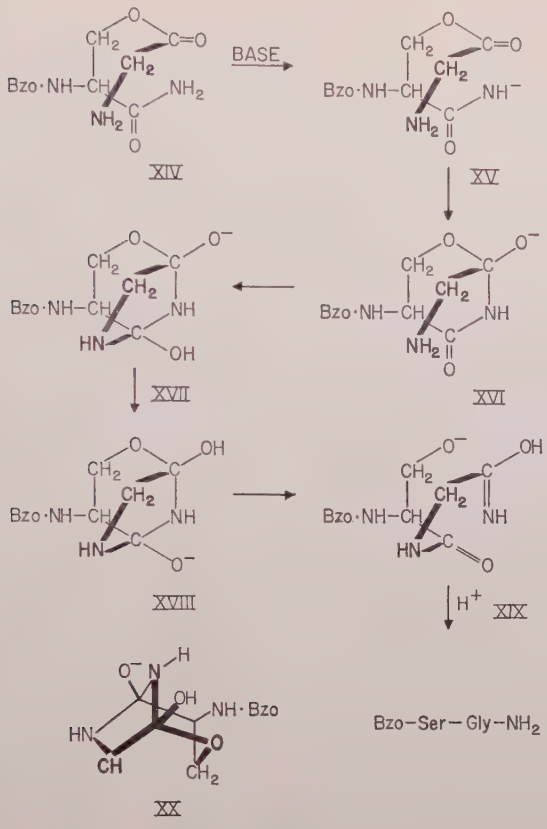

Figure 6

molecular conversion to benzoylserylglycinamide, a reaction type discussed by Wieland and Pfleiderer ('57, see p. 263). In the salicylic acid series, however, there are some findings that favor the assumption of an intermediate of type XVII or XVIII. Formula XX offers a perspective picture of this bicyclic structure. It is analogous to the tropane system. A corresponding intermediate in the general case of intramolecular aminoacyl insertion (fig. 5C) would have the structure shown in figure 7. If b in figure 7 is broken down into two parts, as in figure 5D, it is seen how an activated amino acid or a peptide would have to approach a peptide chain to be inserted at a certain point in that chain.

It will be seen from the theory given of the mechanism that the extrapolations referred to in figure 5 are based on the known capacity of peptide chains to form large-ring compounds. The functions of a good aminoacyl insertion catalyst would therefore be (1) to induce preferential ionization at the required peptide bond, (2) to guide the negatively charged peptide nitrogen to its point of attack (that is, the carbonyl carbon atom of the aminoacyl group to be inserted), (3) to stabilize the cyclic anion so formed, and (4) to guide the N-terminal amino nitrogen of the said

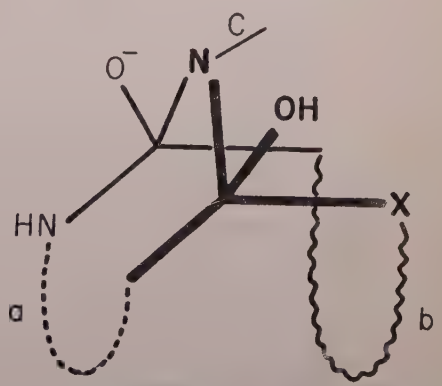

Fig. 7 Generalization of XX; cf. XX, fig. 6. Compare figures 5A, B, and C; X=O, S, or NH.

aminoacyl group on its way to attack the carbonyl carbon atom of the originally ionized peptide bond.

It is not my purpose here to explore the details of the biological mechanism of peptide and protein synthesis. However, a few remarks should be made on the possible biological role of the aminoacyl insertion reaction. For instance, because of its ability to account for ring enlargements without intermediate ring cleavage, the insertion reaction could help explain the existence of certain peptide rings. Second, until now there has been no satisfactory chemical explanation for the observation that there is an interchange of free amino acids with protein-bound amino acids in physiological systems. With the development of the ideas discussed in this paper, this interchange becomes less surprising from a chemical point of view. Now the interchange may be rationally explained by a combination of forward and backward aminoacyl insertion reactions. The alternative process of breaking the chain, interchanging the amino acids at the ends so produced, and recombining the two parts is less attractive because of the low probability of the ends finding each other for recombination. Finally, the aminoacyl insertion reaction can have an effect on theories concerned with the mechanism of protein synthesis. With the possibilities made available by this reaction, it is no longer necessary to postulate that the sequence in a protein is defined in its final form at the time when amino acid units polymerize. Instead, we may imagine that the protein is synthesized in some form resembling its final form and that small changes are then made by an aminoacyl insertion process to bring the protein to its final form. If we pursue this idea to its limit, we may also imagine that amino acid units come together indiscriminately to form a polymer that is then changed to the final form on a catalyst surface by either intramolecular or intermolecular insertion processes.

The multitude of structures given by different combinations of amino acids in peptides and proteins results in a diversity of physical properties unmatched by any other class of chemical compounds. This diversity contrasts markedly with the sameness of chemical reactivity that is a corollary o the mental picture of a polypeptide as string of pearls. The aminoacyl insertio reaction suggests that there is no suc sameness. Indeed it is possible that pep tides and proteins may demonstrate i their chemical properties a diversity equiv alent to their diversity in physical prop erties.

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I also wish to express thanks for th help of Dr. M. A. Stevens in developing th theme of the paper and in preparing th manuscript, and to Mr. H. Curtius, Dr. A Hartmann, Mr. W. Hofer, Dr. H. Kny, Mr H. Moser, Mr. J. Pless, Mr. J. Rosenthale Miss S. Schmidt, Dr. W. Schneider, Dr M. A. Stevens, and Dr. R. Weber for th work that made the development of th ideas in this paper possible.

OPEN DISCUSSION

JENCKS[1]: We have heard about a num ber of stimulating mechanisms for chem ical and enzymic reactions that involve tetrahedral carbon atom from an este amide, or thio ester. The excellent wor by M. L. Bender and others has establishe that such intermediates do exist in este hydrolysis. In most instances in whic such intermediates are known, howeve they are of very high energy and short hal life, indeed. It will be very important i it can be shown that in chemical and en zymic reactions these intermediates d have greater stability and longer half lives Dr. Brenner, do you have evidence tha rules out a mechanism like that of figur 8? In strong base the amide group of th starting material would ionize to a stron nucleophilic reagent and could attack th ester function to make a diacylimide. Dia cylimides are excellent acylating agent and might be expected to undergo a secon intramolecular rearrangement with grea facility.

[1] W. P. Jencks, Brandeis University.

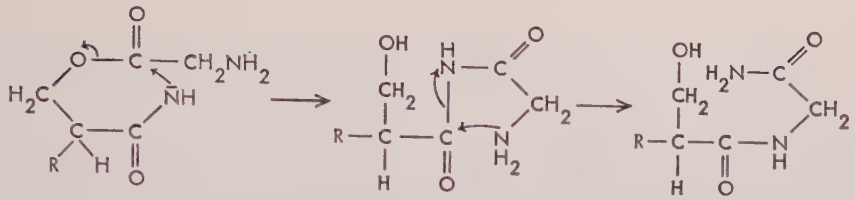

Figure 8

Also, would you say something about the yield that you obtain in aqueous solutions for the peptide rearrangements?

BRENNER: To answer the last question, the yield is practically quantitative in peptide rearrangements. However, it is hard to isolate a quantitative yield of dipeptide amide. The Amberlite hydrolyzes the terminal amide group very easily, and so it is easier to obtain the rearranged dipeptide than the rearranged dipeptide amide. The formation of diacylimide intermediates is a possibility. There are experiments showing that rearrangements of diaminoacylimides to aminoacylamino acid amides do occur. Th. Wieland, of Germany, considered reactions of this type. In his laboratory, diglycylimide was made and he showed that, although it is stable below *p*H 5, it rearranges at *p*H 7 in an intramolecular reaction to give glycylglycinamide. We found that glycylbenzoylimide in the presence of bases gives hippurylamide. Furthermore, the formation of diacylimides may occur through intramolecular migration of an acyl group from phenolic oxygen to neighboring amide nitrogen. We spent a lot of time investigating reactions of this type in the salicylic acid series, and the results are very interesting. For 50 years it has been known that *O*-acetylsalicylic acid amide (fig. 9A) can be made by acetylation of salicylic acid amide. However, if you are not careful you may end up with the *N*-acetyl compound (B). These materials and their transformations lend themselves to ultraviolet and infrared spectroscopic study. When we treat colorless methylene chloride solutions of either A or B with triethylamine we get spectroscopically identical, slightly yellow solutions. In neutral methylene chloride, the two expected infrared carbonyl bands of B are distinctly observed, and, in the case of A, two infrared carbonyl bands also show up, one for the amide and one for the ester group. But in the basic yellow solutions of both A and B, there is only one infrared carbonyl band. On addition of acid to these basic solutions only B is

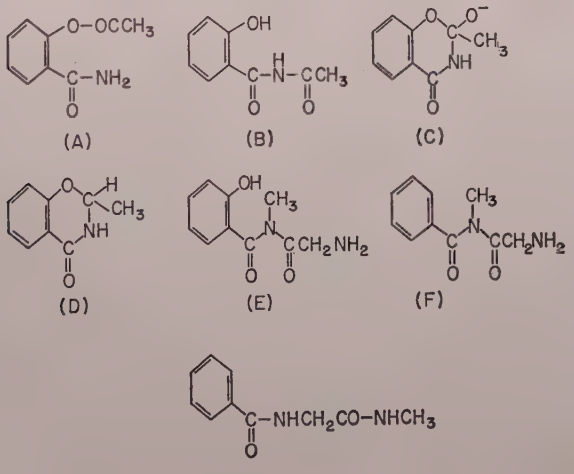

Figure 9

obtained. Assuming that A and B in the presence of base are converted to C, we prepared the similar compound D. The carbonyl bands of the infrared spectra of D and of basic solutions of A and B are at the same place. So we are rather confident that structure C is really present in basic solutions of A and B. Notice that we need an alkaline solution for the aminoacyl insertion reaction to occur. The existence of C makes me assume that there is an analogous intermediate in the aminoacyl insertion reaction. There are other reasons. If we introduce a substituent, any one, into the amide nitrogen of A, then it is no longer possible to demonstrate acyl migration. I cannot explain this. It is merely an experimental fact. However, alkaline solutions are again yellow, and *O*-glycylsalicylic methylamide rearranges to salicylylglycine methylamide in spite of the apparently hindered formation of a diacylimide intermediate (E). Additional evidence against a diacylimide intermediate (E) is furnished by the behavior of F, which is an analog of E. F does not rearrange to give G. Why, then, should E rearrange to salicylylglycine methylamide? Most probably it does not and the actual rearrangement of *O*-glycylsalicylic methylamide does not involve E. These are the reasons for which I assume that diacylimides are not intermediates in the aminoacyl insertion reaction. Whether is is justified to extrapolate the argument to the aliphatic series is, of course, open to discussion. We are still working on that problem.

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The Active Site of Esterases

J. A. COHEN, R. A. OOSTERBAAN, H. S. JANSZ, AND F. BERENDS
Medical Biological Laboratory of the National Defence Research Council T.N.O., Rijswijk Z.H., The Netherlands

This paper presents data from our laboratory pertaining to the chemical structure of the active site of a group of esterases. We attempted to frame these into a general pattern in agreement with well-established knowledge in this field. First we should specify that the term "active site" will refer to those regions of the enzyme surface where the substrate is localized and activated during the enzymic action.

Knowledge of the structure of the active site of esterases is based on indirect evidence mostly obtained by kinetic methods and on direct evidence; the latter usually results from chemical analysis of components of the enzyme protein. The former category will be dealt with only summarily because (1) it has already received much attention by reviewers, (2) it tends to produce results that defy straightforward interpretation, and (3) our personal experience has been limited to chemical methods. Therefore, the evidence based on the application of chemical methods will weigh most heavily in our interpretation of the experimental data.

Among the chemical methods, those involving a specific reaction of the active site of an enzyme followed by the analysis of the groups involved seem the most direct and attractive. Our work was based on such a specific reaction; viz., the property of many esterases to react with diisopropyl fluorophosphate (DFP) to form an enzymically inactive compound. This compound was broken down by proteolytic enzymes, and the structure that carried the diisopropylphosphoryl (DP) residue was analyzed. A rigorous requirement for the validity of the method is the certainty that DFP reacts specifically with the active site. It is now generally agreed that this is so for the following reasons.

The enzymes concerned were inhibited by low concentrations of DFP. Where the molecular weight of the enzyme was known (in trypsin and chymotrypsin), 1 mole of enzyme reacted with 1 mole of DFP to give complete inhibition; correspondingly, when inhibition was only partial, its degree was always linearly related to the amount of DP bound (Balls and Jansen, '52). Other evidence is provided by the well-known ability of substrates to prevent the inhibition by DFP, indicating competition for a common active site. Finally, it is now generally accepted that the inhibition by DFP involves a permanent phosphorylation of the active site analogous to the transient acylation of this site in the course of normal substrate hydrolysis.

Our first results based on this approach (Cohen, . . . Jansz, '55) demonstrated that a number of DFP-sensitive enzymes carry a very similar structure reacting with DFP. We suggested that this common structure (the B group) is closely associated with the general property of enzymic hydrolysis. The substrate specificity of the enzyme, however, would be determined by additional chemical groups on or near the active site. This B group will necessarily consist of amino acids although structures resulting from interaction of amino acid side chains may occur. Results of the continuation of this work will be included.

THE CHEMICAL STRUCTURE OF THE ACTIVE SITE

Imidazole group. Analysis of the influence of *p*H on the rate of hydrolysis by these enzymes of their substrates has shown that an ionizable group of *pK* 6–8 is involved in the enzymic action. Alberty ('55) explored the merits of the method. Similarly, the inhibition of a number of

esterases by organophosphates depends on *p*H. Mounter *et al.* ('57) showed that, for this reaction also, a group of *pK* within this range was essential. Most investigators believe that this group is the imidazole ring of a histidine residue in the active site.

There is also direct chemical evidence for the presence of an imidazole group in the active site (Barnard and Stein, '58; Davies and Green, '58). We shall therefore limit ourselves to a few salient observations in this connection on chymotrypsin (ChTr). Weil *et al.* ('53) and Jandorf *et al.* ('55) showed that destruction by selective photooxidation of one of the two histidines of ChTr destroys the enzyme's activity and its power of combining with organophosphates. Massey and Hartley ('56), Hartley ('56), and Whitaker and Jandorf ('56) have shown that the reaction of 2,4-dinitrofluorobenzene (DNFB) with ChTr reduces the activity of the enzyme in proportion to the extent of the reaction of DNFB with one of its two histidine residues. The value of these results is naturally qualified by the limited specificity of the methods and reagents involved; secondary effects are therefore not excluded.

More support for the significance of imidazole has issued from experiments with model compounds. It could actually be shown that imidazole and its derivatives were, under certain circumstances, capable of accelerating the hydrolysis of esters. The relevant work has been summarized by Dixon, Neurath, and Pechère ('58).

Groups identified in hydrolyzates of phosphorylated esterases. We have emphasized the advantages of DFP as an analytical agent. The attractiveness of the method is based on the good evidence that DFP reacts specifically at the active site. Moreover, it does not drastically change the essential protein structure since removal of the DP group from inhibited enzymes by nucleophilic agents leads to restoration of the enzyme's activity. Results obtained from investigations of the DFP-reactive group in a number of esterases have been discussed by Neurath *et al.* ('59). Since our own work has been exclusively based on this method we shall describe it in somewhat more detail.

The enzyme is first completely inhibited by P^{32}- (or C^{14}-) labeled DFP; sometimes isopropyl methylphosphonylfluoridate (Sarin) is used. Consequently, HCl or proteolytic enzymes (or a combination of both) is used to hydrolyze the isolated P^{32}-labeled protein. The radioactive break-down products have to be separated from unlabeled material; this is done by chromatography and electrophoresis on columns and filter paper. The purified radioactive fragments may then be analyzed. The first study of this kind was undertaken by Schaffer *et al.* ('53) with ChTr inhibited by DFP^{32}. After degradation of the inhibited enzyme with boiling HCl, about 30% of the P^{32} was recovered as serine phosphate. These results have been confirmed and extended by these and other authors. Thus *O*-serine phosphate has been isolated from the following DFP-inhibited enzymes: ChTr (Schaffer *et al.*, '53), aliesterase, and acetylcholinesterase from red cell stroma and pseudocholinesterase (Cohen, Oosterbaan, and Warringa, '55), eel cholinesterase (Schaffer *et al.*, '54), trypsin and horse liver aliesterase (Cohen, Oosterbaan, Warringa, and Jansz, '55). We similarly isolated larger fragments, viz., P^{32}-bearing peptides (P peptides), from enzymes inhibited by DFP^{32} using only mild enzymic hydrolysis with a crude preparation of pancreatic peptidases (Cotazym, Organon, Oss) or with pepsin. They are presented in table 1 together with the results of other authors.

As far as ChTr is concerned, all authors agree on an amino acid sequence (1) of the active site around the P^{32} label, which is invariably attached to the serine residue

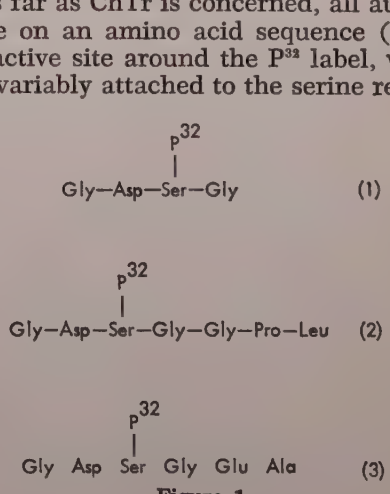


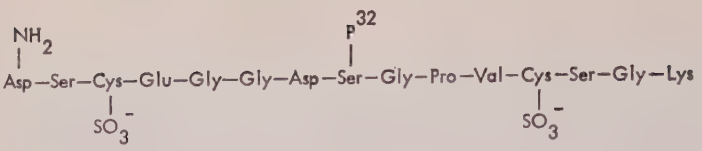
Figure 1

TABLE 1

Amino acid compositions and sequences in the active sites of enzymes

Labeled enzyme	Hydrolysis method	Amino acid composition or sequence	References
Chymotrypsin-DFP	HCl	GLY—ASP—SER(P)—GLY	Schaffer *et al.* ('57)
	HCl	ASP—SER(P)—GLY (Glu, Ala, Gly,)	Turba and Gundlach ('55)
	Cotazym	GLY—ASP—SER(P)—GLY—GLY—PRO—LEU	Oosterbaan *et al.* ('58a,b)
Chymotrypsin-Sarin	HCl	GLY—ASP—SER(P)—GLY—GLU—ALA (Val,)	Schaffer *et al.* ('56, '57)
	Papain	GLY—ASP—SER(P)—GLY—GLU—ALA (Val, His, Pro, Leu, Cys, Thr,)	Schaffer *et al.* ('56, '57)
Trypsin-Sarin	HCl	ASP—SER(P)—GLY	Schaffer *et al.* ('58)
Trypsin-DFP	Trypsin	NH_2-ASP—SER—CYS(SO_3H)—GLU—GLY—GLY—ASP—SER(P)—GLY—PRO—VAL—CYS(SO_3H)—SER—GLY—LYS	Dixon *et al.* ('58a,b)
	Chymotrypsin	Gly,Asp,Ser(P),Gly,Pro,Val,Cys,Ala,Glu,Lys,	Dixon, Go, and Neurath ('56)
	Cotazym	Gly,Asp,Ser(P),Gly,Pro,Val,	Oosterbaan *et al.* ('56)
Liver aliesterase-DFP	Pepsin	GLY—GLU—SER(P)—ALA—GLY—GLY—(GLU,SER,)	Jansz *et al.* ('59a,b)
Pseudocholinesterase-DFP	Pepsin	PHE—GLY—GLU—SER(P)—(Gly,Ala,Ala, Ala,Ser,)	Jansz, Brons, and Warringa ('59) Present authors
Thrombin-DFP	HCl	ASP—SER(P)—GLY and Asp,Ser(P),Gly,Glu,Ala,	Gladner and Laki ('58)
Phosphoglucomutase	HCl or proteolysis	ASP—SER—GLY—GLU—(Ala,Val,Thr,Leu)	Koshland *et al.* ('58); Koshland and Erwin ('57)
Chymotrypsin-NPA	Pepsin and cotazym	$O{=}C{-}CH_3$-GLY—ASP—SER—GLY—GLY—PRO—LEU	Present authors

Remarks: P in the peptide structures denotes the phosphoryl group originating from DFP or Sarin.
GLY—, etc. = amino acid residue in established sequence; GLY, = amino acid residue in unknown sequence;
Gly, etc. = sequence and exact number of residues unknown.



(4)

Figure 2

(fig. 1). Our own results are not in agreement with those of Schaffer *et al.* ('57) as far as the further sequence of larger peptides is concerned. We found sequence (2); the other authors, sequence (3).

As far as trypsin is concerned, the most complete sequence reported by Dixon *et al.* ('58b) [seq. (4), fig. 2] agrees with earlier reports on the composition and structure of smaller peptides as presented in table 1.

Table 1 also includes structures of phosphorylated peptides obtained by HCl hydrolysis of DFP-inhibited thrombin (Gladner and Laki, '58). We have been mainly interested in ChTr, horse liver aliesterase, and pseudocholinesterase hydrolyzed after reaction with DFP^{32} by proteolytic enzymes. Since most of our results with ChTr have already been reported, we shall discuss mainly our recent work with horse liver aliesterase and pseudocholinesterase.

Horse liver aliesterase was purified according to Connors *et al.* ('50). Inhibition with DFP^{32} and peptic hydrolysis yielded a phosphorylated peptide. This was purified as described in figure 3 and analyzed. The amino acid sequence was established as in figure 4. The first two amino acids (N terminal) were identified by the Edman method. The residual P^{32} peptide was refractory against further treatment with phenylisothiocyanate. Therefore, the P peptide obtained from a double application of the Edman technique was treated with DNFB, hydrolyzed with HCl, and *N*-dinitrophenyl-O-phosphorylserine was identified in the hydrolyzate. This is good evidence for attachment of the DP group to the hydroxyl oxygen of a serine residue. The result of the complete structural analysis is represented in table 1.

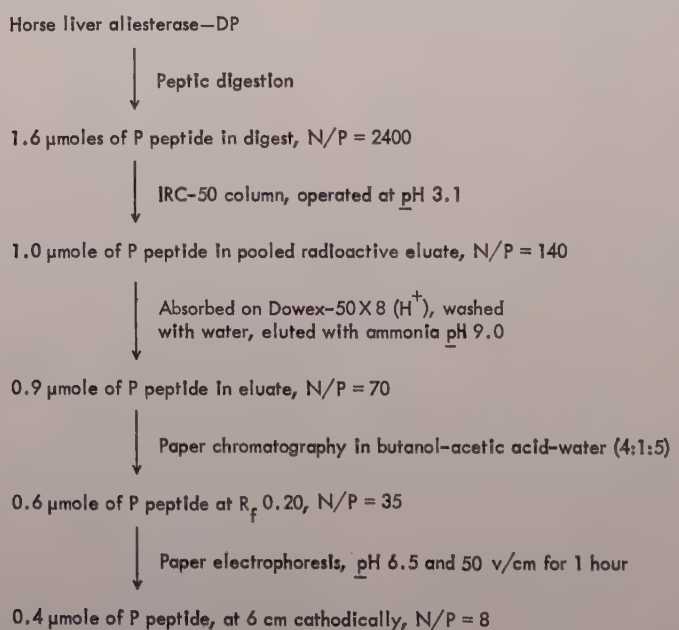


Fig. 3 Isolation of P peptide from a peptic hydrolyzate of DFP^{32}-inhibited horse liver aliesterase. N/P = number of free amino groups per P atom determined in a sample hydrolyzed overnight with 6 *N* HCl at 105°C.

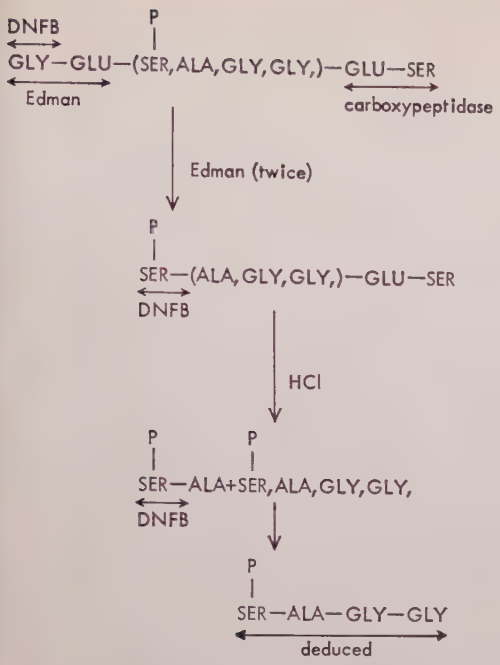


Fig. 4 Elucidation of amino acid sequence of P peptide derived from DFP^{32}-inhibited horse liver aliesterase. The notations of table 1 are used. The C-terminal sequence is based on preliminary experiments.

Pseudocholinesterase (5×10^5 units), representing a 1000-fold purification, was prepared from 700 liters of horse serum by a modification of Strelitz's method ('44). The enzyme was incubated with DFP^{32}, hydrolyzed with pepsin, and the DP^{32} peptide was isolated by chromatography on Dowex-50X4 at *pH* 3.1 and analyzed. Part of its structure could be established by the procedure outlined for the P peptide of DP-aliesterase. Three amino acids from the N terminus were identified by the Edman technique; the identity of the fourth amino acid was established with DNFB (table 1). It is striking that, in contrast to all our previous results, this peptide harbors the P^{32} label in the form of mono-isopropylphosphoryl (MP) instead of DP. The significance of this finding will be discussed later.

We have extended our findings—that the DP group of the P peptide of horse liver aliesterase is attached to the serine oxygen—to the P^{32} peptide isolated from chymotrypsin. All the P^{32} was released as DIP^{32} (diisopropylphosphate) on treating the P^{32} peptide during a few minutes at 100°C. and *p*H 12. Subsequent treatment of the peptide by HCl yielded a hydrolyzate that was devoid of serine. This corresponds with the behavior of *O*-phosphoryl-serine derivatives as reported by Riley *et al.* ('53). These authors showed that alkaline hydrolysis of these compounds results in release of the substituted phosphoryl group accompanied by dehydration of the serine to a dehydroalanine residue. Proof that, in our case, a similar mechanism was operating was obtained by reduction of the dehydroalanyl intermediate in the alkaline-treated P peptide with Pd-H_2 to alanine prior to acid hydrolysis.

Complete hydrolysis of the P peptide by barium hydroxide resulted in a hydrolyzate lacking one serine and one glycine residue but containing one alanine residue instead. This anomaly is also readily explained by dehydration of the P seryl group. The mechanism involved is illustrated in figure 5. The proposed conversion of pyruvoylglycine into alanine has been shown by Fu *et al.* ('52) to occur on alkaline treatment of pyruvoylglycine.

All evidence is in agreement with an attachment of the DP group at the hydroxyl oxygen of the serine residue of the peptides examined. A reasonable inference, therefore, is that all phosphorylated enzymes yielding these peptides in good yield on mild enzymic hydrolysis have the DP group attached to the serine oxygen.

Earlier we stressed the strong evidence available to indicate that DFP reacts with the active site of esterases to produce a stable phosphorylated, inhibited enzyme, whereas the substrates react analogously with the same group to produce a labile acylated enzyme. We were able to prove this point after the work of Hartley and Kilbey ('54), Balls and Aldrich ('55), and Balls and Wood ('56) had shown us the way to isolate the labile intermediate acetyl—ChTr formed during hydrolysis of *p*-nitrophenyl acetate (NPA) by ChTr. Oosterbaan and van Adrichem ('58) prepared C^{14}-acetyl—ChTr by allowing ChTr to react on C^{14}-labeled NPA. The C^{14}—enzyme was first hydrolyzed with pepsin and then with Cotazym. The C^{14} peptides were isolated and analyzed. The complete structure of one of these could be established by con-

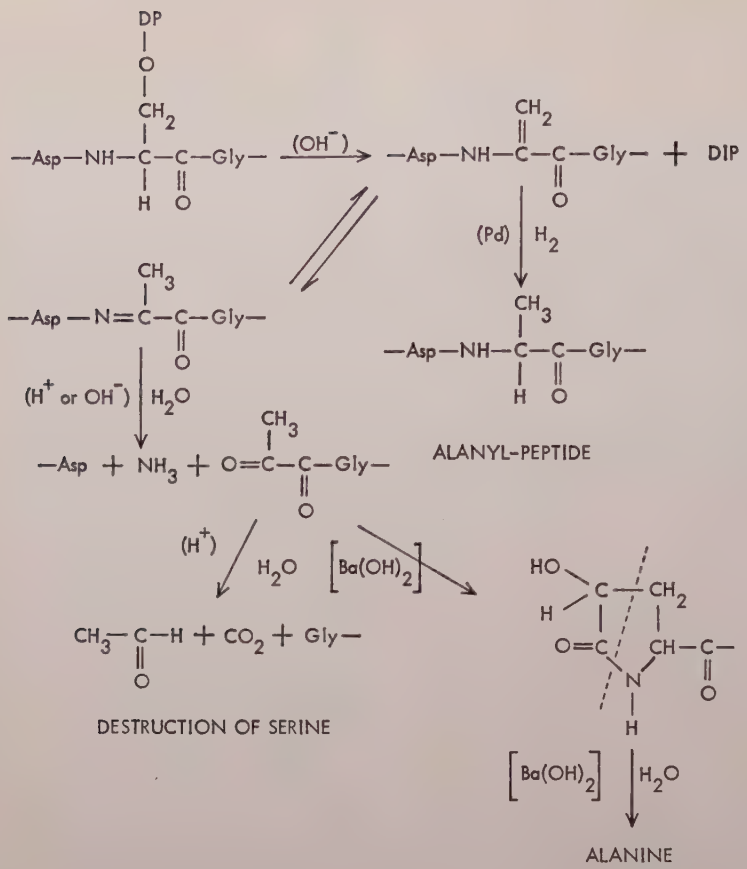
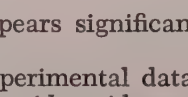


Fig. 5 Fate of serine residue on alkaline treatment of P peptide derived from DFP^{32}-inhibited chymotrypsin.

secutive Edman treatments. It possessed an amino acid sequence completely identical to that of the DP peptide as shown in table 1. The acetyl group is almost certainly attached to the serine oxygen. It is easily released in alkaline solution (*p*H 11). In contrast to what is found with DP-substituted peptides this release is not accompanied by dehydration of the serine residue.

Closer examination of the phosphorylated peptides of ChTr, trypsin, pseudocholinesterase, thrombin, and horse liver aliesterase reveals that the phosphorylated serine residue is always preceded by a dibasic amino acid (Asp or Glu) and followed by a glycine or a closely related alanine residue. Moreover, the dibasic acid is preceded by a glycine residue. Particularly the sequence dibasic amino acid–serine appears significant and not fortuitous.

The experimental data reported in this section provide evidence that, in addition to the amino acid histidine, sequence (5) occurs in the active site of esterases (fig. 6).



(5)

Figure 6

THE MODE OF ACTION OF ESTERASES

We shall now try to visualize the way in which the imidazole, the serine hydroxyl, and the free carboxylic group of the dibasic amino acid residue might operate in the active site of esterases. Such a picture should be consistent with generally

accepted concepts about the mode of action of the enzymes under consideration.

Wilson *et al.* ('50) introduced the hypothesis that enzymic ester hydrolysis might follow the same course as alkaline hydrolysis, in the sense that a nucleophilic group on the active site of the enzyme would play the role of the OH^- ion; it would be acylated during the reaction. Evidence has since accumulated to confirm the occurrence of an intermediate acyl—enzyme (Sprinson and Rittenberg, '51; Wilson, '51; Hartley and Kilbey, '54; Bender and Kemp, '57). Most convincing in this respect has been the work of Balls and Aldrich ('55) and Balls and Wood ('56), who succeeded in isolating acetyl—ChTr from the reaction of ChTr on NPA, and of Dixon and Neurath ('57a), who demonstrated likewise the formation of acyl—enzyme in the course of trypsin-catalyzed ester hydrolysis. Consequently, the following discussion will be based on a mechanism involving the formation of acyl—enzyme accompanied by release of the alcohol residue and followed by hydrolysis of the intermediate to yield acid and free enzyme. Moreover, the assumption seems justified that DFP reacts analogously with the enzyme to form a stable phosphoryl—enzyme. The significance of each of the groups of the active site will be examined against the background of this mechanism.

The imidazole group of histidine. Many workers believe that the imidazole group is the primary acceptor for the acyl or the phosphoryl residue. This concept is strongly favored by the results of model experiments. However, Dixon, Dreyer, and Neurath ('56), followed the reaction between ChTr and NPA spectrophotometrically but were unable to register an increase of the absorption at 245 mμ (the absorption maximum for acetyl—imidazole) during the formation of the acetyl—ChTr. Likewise, Gutfreund and Sturtevant ('56), in studying the same reaction, showed that the acylation does not involve a basic group.

The process of "aging" observed in DFP-inhibited cholinesterases has often been interpreted as evidence for imidazole as the initial site of phosphorylation. The activity of freshly phosphorylated cholinesterase may be restored by the action of nucleophilic agents. Storage, however, produces aging, i.e., the inhibited enzyme gradually loses its ability to be reactivated. The process is usually explained as an intramolecular migration of the phosphoryl group from histidine to an adjacent serine residue (Hobbiger, '55; Jandorf *et al.*, '55). This explanation is consistent with the lability of phosphoryl—imidazole and the stability of *O*-phosphorylserine derivatives. However, aging has been observed only in cholinesterases. Moreover, we found that another mechanism is responsible for the aging process.

We mentioned earlier that the peptide isolated from pseudocholinesterase that had been inhibited by DFP carried an MP instead of the usual DP group. Further studies showed that this MP group was also present in older preparations of DFP-inhibited enzyme; freshly inhibited enzymes carried a DP group. The parallelism with aging is obvious. A systematic study on thoroughly dialyzed DFP^{32}-inhibited pseudocholinesterase gave the following results (Berends *et al.*, '59). (1) During reactivation of the inhibited enzyme by incubation with 0.1 *M* isonitrosoacetone or pyridine 2-aldoximmethiodide (P-2-AM) at *p*H 7.4, part of the P^{32} is released from the protein. The P^{32} thus liberated occurs exclusively as diisopropylphosphate (DIP^{32}); it represents a fraction of the original protein-bound radioactivity that is identical with the fraction of the total enzyme activity recovered (referred to suitable controls). (2) The P^{32}, which cannot be removed by prolonged incubation with the reactivators but remains protein bound, may be released by alkaline hydrolysis (10 minutes at 100°C. and *p*H 12). Under these conditions P^{32} is released as MIP^{32} (monoisopropylphosphate). It cannot result from free DIP or protein-bound DP since, under these circumstances, DIP is stable and DP—enzymes yield invariably DIP. (3) Partially aged, DFP-inhibited pseudocholinesterase consists of a mixture of DP— and MP—enzymes. This may be concluded from the result of alkaline hydrolysis of a sample, which will release all of the P^{32} as a mixture of MIP^{32} and DIP^{32}. The fraction of the original protein-bound P^{32} appearing as DIP^{32} corresponds exactly to the fraction of the original total enzymic activity that may be re-

TABLE 2

Properties of DFP^{32}-inhibited pseudocholinesterase in relation to aging

Time of aging at 24°C.	Alkaline treatment		P-2-AM incubation			P-2-AM incubation followed by alkaline treatment	
	All P^{32} released as:		Restored enzymic activity	P^{32} released as DIP^{32}	P^{32} remaining on the enzyme	All P^{32} released as:	
	DIP^{32}	MIP^{32}				DIP^{32}	MIP^{32}
hr	%	%	%	%	%	%	%
0	95	5	87	94	6	93	7
1	88	12	77	79	21	82	18
2	76	24	63	72	28	71	29
3	67	33	—	—	—	—	—
4	59	41	47	53	47	51	49
6	44	56	36	40	60	38	62
8	33	67	26	29	71	26	74

DFP^{32}-inhibited pseudocholinesterase was dialyzed at 0°C. and kept in a constant temperature bath at 24°C. After the intervals indicated, two samples were taken; one was subjected to alkaline hydrolysis (10 min 100°C., *p*H 12.5), the other was incubated with P-2-AM (0.1 *M*, *p*H 7.4, room temperature). The percentages restored enzymic activity refer to reactivation obtained after prolonged incubation with the oxime.

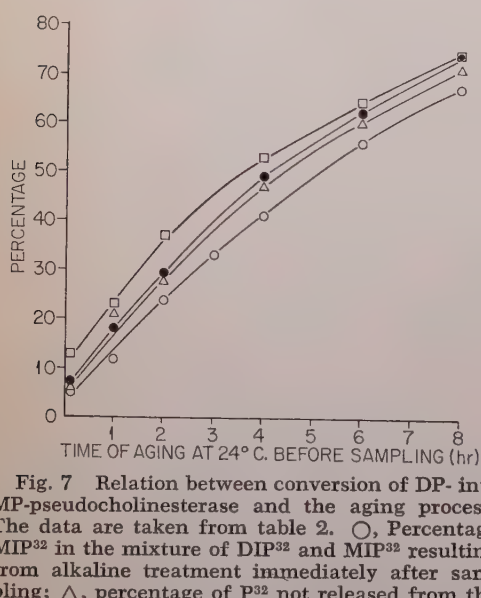


Fig. 7 Relation between conversion of DP- into MP-pseudocholinesterase and the aging process. The data are taken from table 2. ○, Percentage MIP^{32} in the mixture of DIP^{32} and MIP^{32} resulting from alkaline treatment immediately after sampling; △, percentage of P^{32} not released from the protein by prolonged incubation with P-2-AM; ●, percentage of P^{32} released as MIP^{32} on alkaline treatment after prolonged incubation with P-2-AM; □, percentage of enzymic activity not restored by P-2-AM incubation.

trieved by treatment of another sample with reactivators (whereby the same amount of DIP^{32} is liberated). It is obvious that all DP-enzyme is reactivated under release of DIP, whereas MP—enzyme remains intact. Consequently, when reactivators have released all of the protein-bound DP and the preparation is subjected to alkaline treatment, the refractory P^{32} comes off exclusively as MIP.

The results of a representative experiment are summarized in table 2 and figure 7. They show clearly that aging of DFP-inhibited pseudocholinesterase consists in conversion of a reactivatable DP—enzyme into a nonreactivatable MP—enzyme. We have been able to show the existence of a similar mechanism for acetylcholinesterase; aged DFP-inhibited acetylcholinesterase carries an MP group. From studies with C^{14}- and P^{32}-labeled DFP, we learned that, during aging, the isopropyl group came free in the solvent as isopropanol. Under our conditions it is apparently not attached to another site of the enzyme protein.

The data so far presented do not argue in favor of a concept assigning the role of carrier for the acyl or phosphoryl group in acyl— and phosphoryl—enzymes, respectively, to imidazole. On the other hand, the imidazole group may well be involved in reactions leading to acylation or deacylation of enzymes. Kinetic analysis of both processes on the system ChTr—NPA demonstrated the importance of a group with *pK* 6–8 (Dixon and Neurath, '57a). Little or nothing is known about how imidazole may influence the acylation. The role of imidazole in deacylation seems fairly well established since Dixon and Neurath ('57b) have shown spectrophotometrically that deacylation of acetyl—ChTr is accompanied by the formation of

an acetyl—imidazole intermediate. T. Viswanatha (private communication) reports that he has succeeded in isolating enzymically active peptides from peptic hydrolyzates of acetyltrypsinogen. These peptides were devoid of histidine (not more than 0.1 mole of histidine per mole of peptide in good preparations). These results may be of great interest for the final evaluation of the significance of histidine.

The serine hydroxyl. Attachments of phosphoryl groups at a serine hydroxyl of inhibited enzymes has long been considered an artifact. It was thought that, in the course of degradation, isolation, or aging, the phosphoryl group would migrate from the active site to a serine residue. Consequently, the significance of serine phosphate and phosphoryl peptides isolated from inhibited enzymes seemed doubtful. It soon became apparent, however, that from many different esterases very similar phosphorylseryl peptides (and also acetylseryl peptide from acetyl—ChTr) could be isolated by a variety of methods (HCl, various proteolytic enzymes). These results have established the significance of the serine residue at the active site; moreover, the aging process can no longer be taken as evidence for migration. The conclusion therefore seems justified that in these esterases the serine hydroxyl functions as acyl and phosphoryl acceptor.

This conclusion implies a high reactivity of the seryl hydroxyl and this high reactivity remains unexplained by the present knowledge of the active site. The reason for it may be that serine is incorporated in a special structure, e.g., by cyclization to a Δ^2 oxazoline ring as suggested by Porter *et al.* ('58) (fig. 8). They showed that Δ^2 oxazolines may react with DFP to give products that yield *O*-phosphorylethanolamines on acid hydrolysis. Another possibility would be that other groups, on the strength of their steric position, could induce high reactivity in the serine hydroxyl. Westheimer ('57), Brouwer ('57), and Cunningham ('57) suggested that imidazole might be in such a position. Cunningham ('57) proposed a detailed mechanism for the enzymic action of ChTr consistent with data available at the time (*pK* for acylation and deacylation, *p*H and denaturation stabilization of acyl—enzyme and occurrence of acyl—imidazole during deacylation).

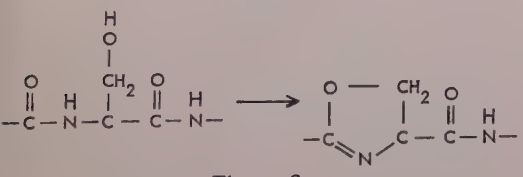


Figure 8

The carboxyl group. As discussed before, the available evidence on the chemical structure of the active site favors the concept that the amino N of the essential serine is linked to the α-carboxyl of a dibasic amino acid residue. It seems likely that this sequence (Asp—Ser or Glu—Ser) is an obligatory property of the active site. It will therefore be necessary that models explaining the enzymic action of the esterases under consideration take account of this residue and its function, e.g., the presence of a functional free carboxyl group in the right position. Reaction schemes and models that fail to account for this group seem to us less satisfactory.

The problem that arises is to determine the significance of this residue with regard to the functional characteristics of the active site, viz., substrate hydrolysis and reactivity toward organophosphates. For instance, the free carboxyl group of the dibasic amino acid could serve as acceptor for the substrate alcohol like serine serves as acceptor for its acyl residue.

It is conceivable that in the active enzymes the serine hydroxyl and this carboxyl group are linked to give an internal ester. Substrate hydrolysis would then involve transesterification; in this process the imidazole group could take part, e.g., by promoting acylation or deacylation.

A second possibility accounts for reported properties of the carboxyl group with regard to ester hydrolysis. Several authors (Edwards, '50 and '52; Garrett, '57; Bender, Chow, and Chloupek, '58; Zimmering *et al.*, '57; Morawetz and Oreskes, '58) demonstrated that an internal carboxylate ion in the right position relative to the ester bond may participate catalytically in the hydrolysis. The hydrolysis of acetylsalicylic acid may be regarded as a classical example. The acceleration of

the hydrolysis is almost certainly attributable to an intramolecular attack of the carboxylate ion on the carboxyl carbon atom of the ester to produce an intermediate (mixed) anhydride, which is subsequently rapidly hydrolyzed. Bernhard and Gutfreund ('56) proposed that catalysis by the enzyme ficin is dominated by a group with a *pK* of 4.35, presumedly an ionized carboxyl group rate determining for the breakdown of an acylthiol—enzyme compound; similarly, Smith and Parker ('58) demonstrated that in papain a carboxylate ion participates in the hydrolysis of the thiol ester intermediate. Bender, Chloupek, and Neveu ('58) suggested that the mixed anhydride mechanism for intramolecular catalysis operates during the action of such —SH enzymes. We should like to extend the hypothesis to include the intermediate *O*-acylseryl—enzymes formed during esteratic action. The significance of the dicarboxylic acid residue would thus be the furnishing of a carboxylate ion in a steric position suitable to enable it to catalyze the hydrolysis of either *S*-acylcysteyl (in the case of —SH enzymes) or *O*-acylseryl formed during esteratic or proteolytic action; the catalytic action would consist of a nucleophilic attack on the ester carboxyl C atom leading to a labile acid anhydride. If this hypothesis is true, it would involve the presence of a dicarboxylic amino acid residue not only preceding the serine residue in the active site of esterases but also the cysteine residue in the active site of proteolytic —SH enzymes.

The role thus assigned to the dicarboxylic acid is limited to the last phase in the enzymic process, the hydrolysis of the acyl—enzyme complex.

Attempts to relate the reactivity of serine hydroxyl groups with the free carboxyl group of the active site are still very speculative. Smith ('58) suggested that the reactive group at the active site of papain involves the internal thiol ester in which the free carboxyl group participates.

PERSPECTIVES

We should now like to try to assess the significance of this work and of chemical knowledge about active sites in general with regard to biological reactions.

It should first be realized that our meth od is limited to enzymes inhibited by DFP Our conclusions therefore are valid onl with regard to the active sites of many though by no means all, esterases and c only some of the proteases that posses esterolytic properties. It should be recog nized that DFP-nonsensitive esterases an proteases may possess an active site c quite a different chemical constitution Some esterases hydrolyze DFP. It may b that these closely resemble the B grou possessing enzymes, the only essential dif ference with the latter being lability rathe than stability of the phosphorylated en zyme.

A number of proteases (e.g., cathepsins papain, ficin) have an —SH group in thei active site. They are insensitive to DFP They may still possess a structure analo gous to the B group with cysteine occupy ing the place of serine. It may be tha cysteine —SH embedded in a B-grou structure has a function analogous to tha of serine hydroxyl in proteolysis.

Thus it seems that the B group or a analogous structure may have a wide spread occurrence in nature: it has bee found in many hydrolytic enzymes. It significance is therefore presumed to b closely related to the activity of hydrolyti enzymes.

It should be remembered, however, tha the enzymes studied, crystallized or othe wise purified, are often the sorry results o a series of maltreatments to which a pro tein has been subjected on its way fro its natural biological position in harmon with the total of the organism into our tes tube. Therefore, the obvious conclusio that *in vitro* hydrolytic ability represent the only *in vivo* function of isolated pro teins is not always tenable and has ofte proved wrong. It is certainly warrante for proteins like ChTr and trypsin tha occur in the intestinal tract of animal where they break down proteins into me tabolites that are readily absorbed into th general circulation. Correspondingly wit regard to the *in vivo* function of these en zymes the significance of the B group wi be that of a structure arranged in such way within the enzyme macromolecul that it favors the hydrolysis of substrate

In this process the macrostructure is acylated at the serine hydroxyl site followed by deacylation, i.e., transfer of the acyl group to the acceptor, water.

It is certain that a number of enzymes that *in vitro* hydrolyze substrates act *in vivo* as transferring enzymes owing to the presence of acceptors other than water. In this connection, Koshland and Erwin's suggestion ('57) that a nonhydrolytic enzyme, phosphoglucomutase, possesses the B-group structure at its active site is of great interest. These results provide chemical support for the concept that hydrolases may be considered as a special class of transferases. The general mechanism of all these enzymes is acylation or phosphorylation of the serine hydroxyl at the active site followed by transfer of the acyl or phosphoryl group to an acceptor, which may be water, an alcohol, or something else.

In other cases isolated proteins with hydrolytic action *in vitro* may *in vivo* have enzymic activities other than hydrolysis or transfer; they may *in vivo* even be endowed with other than enzymic properties of high biological significance. The muscle protein myosin is a good illustration of this.

Likewise, it may very well be that the main *in vivo* function of acetylcholinesterase, an enzyme found in cholinergic receptors and in the membrane of erythrocytes, is not the breakdown of acetylcholine. A number of authors (e.g., Holland *et al.*, '52; Berman *et al.*, '53) have suggested that the function of acetylcholinesterase is associated with permeability processes. Interaction of acetylcholine with acetylcholinesterase protein embedded in receptor structures may lead to the triggering of changes of permeability in those structures. It is also conceivable that *in vivo* some esterases may be involved in permeability processes in the sense that they may be able to transfer the acyl group of esters through membranes, passing it first toward receptors and from there onward. Similarly, they may promote the passage of the alkoxy group of esters. Stein ('58) showed that a polypeptide of the red cell membrane may be essential for the permeation of glycerol; the amino terminal histidine of this peptide was essential. A relation with the imidazole of the active site of esterases might be considered in this connection. Clearly, studies of the active site of these esterases may thus provide information on permeability of membranes.

The reactivity of enzymes with their substrates, inhibitors, or activators is usually striking with regard to specificity and affinity at physiological *p*H, temperature, and pressure, and the same applies to the reaction of biologically active metabolites or drugs with their respective receptors. The chemical structure of the active site of an enzyme capable of reacting with a biologically active compound represents at least one way in which the living organism might be able to react with the latter. It therefore seems likely that an organism containing an enzyme that reacts with a biologically active compound will possess receptors for it that bear likeness to the active site of this enzyme. These receptors will be the primary sites of attack of the drug when it elicits its biological effect. Thus the active site of atropinase occurring in rabbit serum may carry information on the receptors for atropine in the organism, the active site of penicillinase may reveal the way in which penicillin attacks the sensitive sites in bacteria, and that of histaminase the way in which histamine interacts with its numerous receptors in animals. The chemical structure of the active site of DNase may be revealing with regard to the role played by DNA in the passing of genetic information.

Seen in this perspective, the results reported in this symposium, primarily undertaken to elucidate enzymic action, might prove of paramount importance for the understanding of the action of numerous drugs and metabolites.

OPEN DISCUSSION

HARTLEY[1]: Dr. Cohen can you remove the acetyl group from serine in peptide, and will the peptide then react with *p*-nitrophenyl acetate?

COHEN: You can get the acetyl off without interfering with the structure of the peptide, but the deacetylated peptide does not react with *p*-nitrophenyl acetate.

VISWANATHA[2]: Dr. Cohen, since you have observed the same sequence around the active site with quite a few enzymes, would you comment on the basis for the differences in specificity of these enzymes.

COHEN: We should not be dogmatic about this active site. First of all our B group is probably only part of the active site. The idea we have is that the enzymes concerned share a common denominator that is part of the active site and the substrate specificity is conveyed to the enzyme molecules by quite a number of additional properties in which they differ.

VISWANATHA: The change in specificity observed in the case of an active derivative from acetyltrypsinogen tempted me to raise the foregoing question. If the secondary and tertiary structure of the enzyme molecule were to play a role in determining its specificity, I wonder whether partial elimination of these structures by degradation would result in a lowering of the specificity of the enzyme.

[1] B. S. Hartley, University of Washington.
[2] T. Viswanatha, National Institutes of Health, Bethesda.

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Enzyme Flexibility and Enzyme Action[1]

D. E. KOSHLAND, JR.
Biology Department, Brookhaven National Laboratory, Upton, New York

It has long been clear that enzyme action is intimately involved with the three-dimensional arrangement of amino acids. The specificity of the enzymes led Fischer (1894) to propose a "key-lock" model for the steric relations at the active site, and others showed that denaturation was correlated with changes in shape of the enzyme. The tools for measuring protein shapes are still primitive, and the physical description of that ill-defined area called "the active site" is even more complicated. Nevertheless, unsuspected tools are frequently uncovered under the impetus of pertinent questions and we should like, therefore, to attempt an answer to the question "Is the active site rigid or flexible during enzyme action?".

One source of information comes from the general studies on protein shape, which have followed almost from the discovery of proteins and protein denaturation. The first evidence that proteins were flexible as well as fragile probably comes from the studies of Anson and Mirsky ('34) on the reversible denaturation of trypsin. Since then the able and original work of a number of workers (e.g., Karush, '50; Kauzmann, '54; Lumry and Eyring, '54; Doty and Yang, '56; Linderstrøm-Lang and Schellman, '59) has led to a far greater understanding of this vital area. In addition to temperature, other reagents such as urea, *p*H, salt concentration, and organic solvents can be used to induce changes in the three-dimensional geometry of a protein. The changes in shape caused by these stresses can be measured by a variety of tools of which optical rotation, viscosity, sedimentation constant, deuterium exchange, and solubility are examples. A brief and over-simplified summary of all of this work is that certainly large portions of many proteins are flexible in the sense that they can be reversibly deformed. Urea, for example, will produce reversible changes in viscosity and enzyme activity in both trypsin and chymotrypsin (Harris, '56). With almost all these reagents, there appears to be a point of no return, after which irreversible changes are induced. If this limit is not exceeded, however, the evidence supports the conclusion that removal of the stress returns the flexible portions of the protein to their natural conformations.

The fact that large portions of the protein are flexible is by no means evidence that all portions of the protein are. Actually, fragmentary evidence exists that a change in certain portions of the protein always results in irreversible denaturation. We are left, therefore, with the conclusion that either a flexible or a rigid active site would be compatible with the general studies of protein properties.

Let us, therefore, examine the evidence for the template model of enzyme specificity that argues for a relatively hard and inflexible active site. To a chemist, one of the most astounding properties of enzymes is their specificity. The fact that a small group in the substrate, far from the bond to be cleaved, can decide whether an enzyme acts or does not act on a particular compound is almost incredible. Since studies of physical organic chemistry have clearly indicated the magnitude of inductive effects, we can conclude that such effects cannot explain the observed changes in velocity from substrate to non-substrate in most cases. Fischer therefore proposed the template model with which he postulated that the substrate must be able to fit on the surface of the enzyme in order to get in close proximity with the catalytic groups there. If the groups

[1] Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

were too bulky to allow this association, no catalysis resulted. If the groups necessary for binding the substrate to the enzyme were absent, the substrate was not held to the enzyme and again no catalysis would result. Since the two postulated phenomena for the template hypothesis, i.e., steric hindrance and affinity by the formation of noncovalent complexes, were well substantiated in organic chemistry and since the development of enzyme kinetics supported the presence of an enzyme—substrate intermediate, this theory became widely accepted. In fact, it does suffice to explain the vast majority of the observed specificity patterns of enzymes.

Our own feelings that all was not quite so well with the template theory as might appear on the surface came when we were trying to explain the failure of muscle phosphorylase to catalyze an exchange between $P^{32}O_4$ and glucose 1-phosphate (Koshland, '54). In muscle phosphorylase, it had been shown that an acceptor was needed to observe exchange whereas with sucrose phosphorylase no acceptor was required (Doudoroff *et al.*, '47; Cohn and Cori, '48). Let us assume that the same mechanism is operating for muscle phosphorylase as was indicated for the sucrose synthesizing enzyme; i.e., that a group on the enzyme attacks from the back of the carbon atom to form a glucosyl—enzyme intermediate. The existence of this mechanism does not necessarily mean that exchange must occur. It could be said that the glucosyl—enzyme intermediate exists for so short a time that the inorganic phosphate is unable to leave and be replaced by a radioactive phosphate before the new covalent glucose—phosphate bond is formed. There is good analogy for this kind of kinetic variation in the neighboring group effect, in which the gamut from the formation of a completely stable bond, as in epoxides, to the transient interaction of a neighboring methoxyl group is observed. However, if such a process were going on and there were repeated formations of a glucosyl—enzyme intermediate, we might expect that periodically the water in the adjacent site would be able to react. Water should certainly be about as nucleophilic as the 4-hydroxyl group of the glycogen polymer, and it seemed unlikely that the glucosyl—enzyme intermediate being formed so rapidly and reversibly would not occasionally react with the adjacent water molecule. An alternative mechanism based on the S_{N1} reaction (Koshland, '54) leads to almost precisely the same difficulty.

It would seem that either the displacement mechanism or the template theory was inadequate. This would hardly be sufficient basis for questioning the template hypothesis, but on reflection we thought the failure of water to react in a number of other instances (e.g., the hexokinase reaction) was equally puzzling. Moreover, evidence in support of the displacement mechanism increased, and an intensive search of the literature was therefore made for examples that could not be reconciled with the template hypothesis. An amazingly large number of instances were found (Koshland, '55, '58, '59), and since this material has already been published, only one example will be used to illustrate the type of reasoning involved.

Amylomaltase is a purified enzyme that catalyzes the hydrolysis of maltose but does not act on α-methylglucoside (Wiesmeyer and Cohn, '57). α-Methylglucoside has the same stereochemistry at the C-1 as maltose and the same type of bond to be broken; it differs only in that the methyl group has two hydrogen atoms where the remaining part of the second glucose ring would be placed. Since it could hardly be argued that these two hydrogen atoms would be unable to fit into the area on the template reserved for the full glucose ring, the failure of α-methylglucoside to react would, on the template hypothesis, have to be explained by a failure to be attracted to the enzyme surface. However, α-methylglucoside has been shown to be a competitive inhibitor. Hence it is known to be present at the enzyme surface and in the appropriate position, and yet no reaction occurs.

From examination of these and other examples, it was clear that the template theory would have to be modified. The reasoning that led Fischer to conclude that a steric interaction was required seemed unassailable. The theory was modified, therefore, to give the substrate a more-

positive role. It was assumed that the active site was not initially a negative of the substrate but became so only after interaction with substrate. This change in conformation of the protein occurred with the result that the final enzyme—substrate complex had the catalytic groups on the enzyme in the proper alignment with each other and with the bonds to be broken in the substrate molecules. This retained the idea of a steric fit proposed by Fischer but modified it in such a way that the failure of either too large or too small a compound to react could be readily explained. For example, the failure of water to react in the phosphorylase reaction would be explained by the fact that the small size of the water molecule did not provide sufficient buttressing action to lead to the proper alignment of catalytic groups. This, moreover, is in line with the observation of a minimum size for the primer in the phosphorylase reaction. This mechanism requires a flexible action at the active site, i.e., the protein changes shape under the influence of the substrate and returns to its original shape after the products have been released from the enzyme surface. This "induced fit" hypothesis also explained a number of other observations such as the synthetase-type enzymes that require the simultaneous presence of a number of substrates on the enzyme surface before any partial reaction occurs (Koshland, '55, '58, '59).

Some evidence of a different nature obtained by Dr. Harvey Levy and Dr. Nathan Sharon (Levy *et al.*, '59a) supports the postulated flexibility of the active site and the specific modification of protein conformation by the substrate itself. This evidence grew out of temperature studies on the enzyme myosin, the data for which are shown in figure 1. Recording the rate data on an Arrhenius plot gives a straight line if the activation energy and the PZ factor are constant. Such is observed to be the case for the myosin-catalyzed hydrolysis of ATP, which is linear over the experimental range of 30° to 0°C. It is to be noted, however, that both ATP in the presence of dinitrophenol (DNP) and ITP show a pronounced curvature. This is not a function of the experimental error or of the method of graphing. Careful repeti-

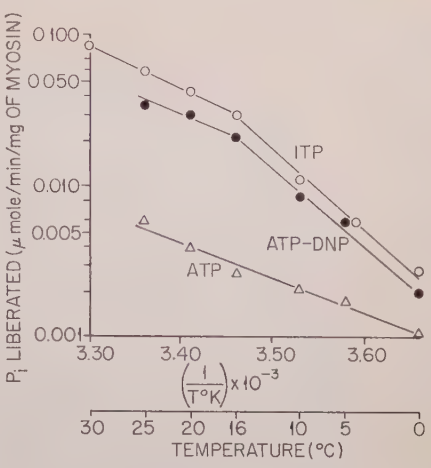

Fig. 1 Arrhenius plot for myosin with ATP, ITP, and ATP in the presence of DNP. Solution contained 0.01 *M* $MgCl_2$, 0.05 *M* Tris, 0.1 *M* KCl *p*H 7.3, and 0.005 *M* nucleotide triphosphate.

tion of the experiment and replotting of the data with various abscissa and ordinant ratios leads to the same conclusion. Actually, the two curves can be very well approximated by straight lines at each of the extremes of temperature, and these straight lines intersect in each case very near 16°C. This extrapolation should not be taken to imply that an abrupt discontinuity exists. However, the rather good agreement of the straight lines over a considerable range of temperature does tend to indicate that a shift occurs from a process at the higher temperature having an activation energy of ~12 kcal to a process at lower temperatures having an activation energy of ~25 kcal.

Curves that show such a change in activation energy have been observed before, and a number of different explanations have been proposed. Dixon and Webb ('58) have summarized these as follows.

(a) There is a phase change in the solvent. This idea is supported by the observation that the point of inflection apparently occurs in the same place for a number of different enzyme systems and by the existence of a transition point near 0°C. where a phase change in the solvent water is known to occur.

(b) There are two parallel reactions with different active centers. Dixon and Webb ('58) pointed out that such a mechanism could explain Arrhenius plots that

are concave upward but could not explain those that are concave downward.

(c) The enzymic process involves two successive reactions having different temperature coefficients. The idea that such a shift from one rate-mastering step to another was responsible for the transition in the Arrhenius plot of physiological processes was originally suggested by Crozier ('24) but was later seriously attacked by Burton ('39) and others. Burton showed that, in some systems having activation energies of the order of those found by Crozier, a consecutive-step mechanism would not lead to so abrupt a transition as was observed in the experimental cases. Burton was dealing with a system involving two different enzymes, however, and the mathematics accordingly is not precisely the same as that for two consecutive steps on a single enzyme surface. The sequence of events in the latter case are illustrated by equation (1) (fig. 2). Since the experiments reported here were done at enzyme saturation, we need only consider the steps in equation (1) having the constants k_1 and k_2. When the kinetics for this case are derived by using only the steady-state assumption and the condition that $(ES)_1 + (ES)_2 = E_T$, the relation shown in equation (2) is obtained. This turns out to be different from the kinetics treated by Burton; the observed values for

$$E + S \rightleftharpoons (ES)_1 \xrightarrow{k_1} (ES)_2 \xrightarrow{k_2} E + P \qquad (1)$$

$$\frac{d(P)}{dt} = \frac{k_1 k_2 E_T}{k_1 + k_2} \qquad (2)$$

$$(E_1S) \xrightarrow{k_1} E_1 + P \qquad (3)$$

$$E_1 \overset{K_1}{\rightleftharpoons} E_2 \qquad (4)$$

$$(E_2S) \xrightarrow{k_2} E_2 + P \qquad (5)$$

$$\frac{d(P)}{dt} = \frac{k_1 K_1 + k_2}{1 + K_1} \qquad (6)$$

Figure 2

an enzyme having activation energies similar to those of myosin with ITP are shown in figure 3. Since the observed rates for such a consecutive-step mechanism are seen to be a very good rough approximation for the ITP-myosin curve, it is clear that a consecutive-step mechanism cannot be excluded simply by the argument that the transition observed experimentally is too abrupt.

(d) The enzyme exists in two forms having differing activities. This suggestion was originally advanced by Sizer ('43) and can be formalized as shown in equations (3), (4), and (5). In this mechanism the protein changes from a high-temperature form, E_1, to a low-temperature form, E_2, in a reversible manner over a fairly narrow temperature range. The velocity of the over-all reaction is given by equation (6). This mechanism can give Arrhenius plots that are either concave

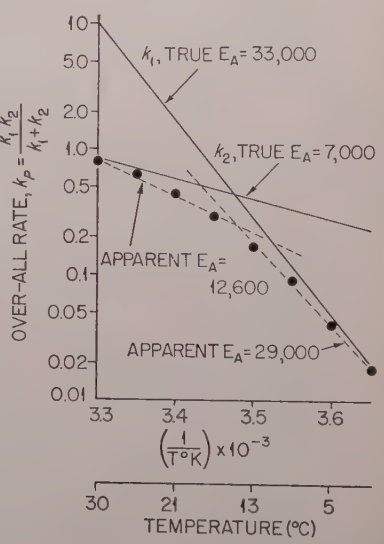

Fig. 3 Calculated velocities for a consecutive-step mechanism of the type shown in equation (1) (fig. 2). The true activation energies of k_1 and k_2 are assumed to be 33 and 7 kcal, respectively. The absolute magnitudes of the rate constants for these two steps are taken from the solid lines drawn with these activation energies. The rate of appearance of product at any temperature is obtained from these values by equation (2) and is shown by the points on the graph. These velocities, which would be the observed velocities in an experimental case, give apparent activation energies of 12.6 and 29 kcal in two rather linear portions of the curve.

downward (see fig. 4) or concave upward (see fig. 5).

(e) There is a reversible inactivation of the enzyme. Kistiakowsky and Lumry ('49) explained a transition in the urease curve by a reversible inactivation involving sulfite. In a sense this is different from the previous case because an added reagent is involved. In the absence of added inhibitor, inactivated enzyme can be viewed as a special case of a changed enzyme in which k_2 happens to be zero.

(f) There is a discontinuity affecting the forward reaction only. This alternative is advanced to explain the case of fumarase (Massey, '53) that shows a transition for the forward reaction but none for the reverse.

By combining the data of figure 1 with the literature, it can be shown that all these alternatives are unlikely if a template-type specificity is required. Detailed arguments will be presented elsewhere (Levy, Sharon, and Koshland, unpublished) but a typical line of reasoning can be presented here. It involves the assumption that a single explanation will suffice for all cases in which a transition is observed in the Arrhenius plot.

Thus the myosin behavior clearly excludes alternatives (a) and (e). In each of these cases, it is postulated that an external change occurs (i.e., a solvent change or protein denaturation) that is independent of the substrate and hence should affect the two substrates in a qualitatively similar manner. It is, of course, not necessary that the solvent change affect the two rates equally, but it could not dramatically change the velocity of the ITP hydrolysis without making at least some change in the ATP rate. A protein inactivation would, of course, result in a similar effect in the two cases. Alternative (f) is excluded in the present case since both substrates are operating in the forward direction and alternative (b) is excluded because the ITP is concave downward. Alternative (c) is compatible with the ITP data but is inconsistent with the fumarase data (Massey, '53).

This leaves alternative (d), the protein change mechanism. If this enzyme obeys template-type specificity, this alternative can be excluded by the same arguments used to exclude alternatives (a) and (e). Whether the new form of the protein is active or inactive, a transition for one substrate should be accompanied by a transition for the other. This conclusion is predicated on two experimental condi-

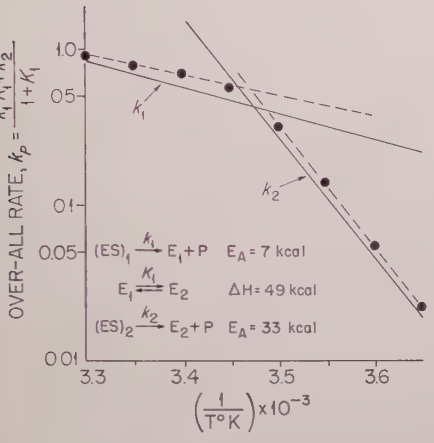

Fig. 4 Calculated velocities for the protein change mechanism. Theoretical rates of product appearance are calculated for the mechanism shown in equations (3–5) by using an activation energy for k_1 of 7 kcal and for k_2 of 33 kcal. The ΔH for the protein transition was taken to be 49 kcal with the assumption that $E_1 = E_2$ at 16°C. Values for the observed rate at any temperature are obtained by solving equation (6) (fig. 2) when values for k_1 and k_2 taken from the solid lines in the figure are used.

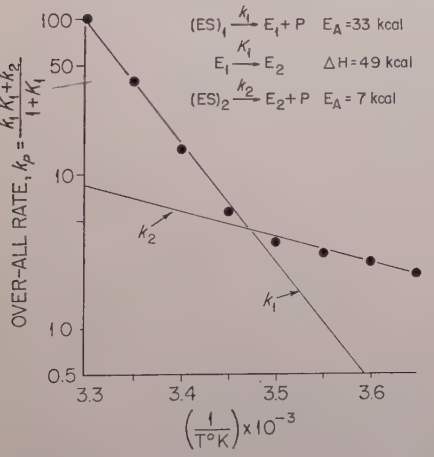

Fig. 5 Calculated rates for the protein change mechanism. Same procedure as for figure 4 except that the activation energy for k_1 is assumed to be 33 kcal and for k_2 7 kcal. The ΔH for the E_1 to E_2 protein change is the same as for figure 4.

tions: (i) that we are dealing with saturated enzyme and (ii) that the two substrates are competing for the same site. Both these conditions are true in the present case. The enzyme saturation was shown by the usual type of kinetics, and the competition for the same active site was shown by the hydrolysis of ATP^{32} in the presence of nonlabeled ITP. It is of interest that in the latter experiment the ITP, which by itself is hydrolyzed ten times as fast as ATP, is not hydrolyzed at all in the presence of ATP because of the far greater binding affinity of the latter substrate.

For those who distrust complicated arguments, it may be worth emphasizing that the qualitative difference in Arrhenius plots of ITP and ATP is by itself difficult to explain by a template-type specificity. The 6 position in the purine ring is many bonds removed from the bond being split in the enzymic reaction. The —OH and $—NH_2$ groups both have an unshared pair of electrons, both have a hydrogen available for hydrogen bond formation, and both have very similar volumes. A change in the template that dramatically affects the decomposition of the enzyme—ITP complex without visible effect on the enzyme—ATP complex is difficult to imagine.

The protein change mechanism, however, can explain the existing facts if a flexible active site having different conformations in the presence of ITP and ATP is assumed. A schematic illustration of such an interaction is shown in figure 6. At the higher temperature the enzyme exists in a rather loose structure, with a chain of considerable length leading from the active site. It is assumed that there is a group X in this chain that is strongly attracted to the 6-amino group of ATP but has no affinity for the 6-hydroxy group of ITP. This attraction leads to a constriction of the amino acids at the active site when ATP is adsorbed and this constriction is presumed to make the active site less favorable for enzyme action. Accordingly, the ITP is hydrolyzed far more rapidly than ATP. When the enzyme is cooled, a change occurs leading to a coiling in an area adjacent to, but not immediately at, the active site. This coiling, however, shortens the chain leading from the active site so that it no longer has the flexibility at the lower temperature that it had near 25°C. This coiling, which proceeds rather abruptly in the region of 16°C., therefore causes a marked decrease in the freedom of the ITP active site with concomitant change in enzyme activity. However, the same coiling has essentially no effect on the activity of ATP since the 6-amino group attraction already has caused a constriction of the active site. Thus the folding of the protein (perhaps into an α-helix) has different effects on ITP and ATP hydrolyses because the interaction of the two substrates with the protein does not result in identical protein conformations.

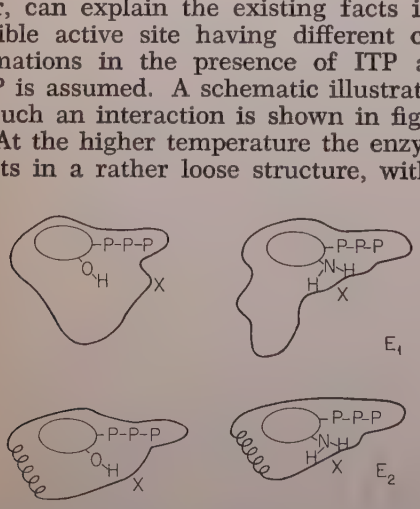

Fig. 6 Schematic illustration of induced-fit behavior to explain the temperature dependence of ITP and ATP in myosin hydrolysis.

This model is illustrative and is delineated here mainly to show that the induced-fit type of behavior can explain why two substrates can have qualitatively different temperature dependences. Nevertheless, there is considerable added information that it is a good model for the action of myosin. First, the difference between ITP and ATP does not establish whether the —OH or the $—NH_2$ group is playing the positive role. In further studies, tripolyphosphate gave a curve similar to that of ITP, showing that it is the absence of the $—NH_2$ group rather than the presence of the —OH group that is responsible for the difference. That the tripolyphosphate curve shows a transition at 16°C. similar to ITP further establishes that it is not a difference in size between $—NH_2$ and —OH that is responsible for the transition. Second, the role of DNP in this system can be readily explained with this model. DNP makes the hydroly-

sis of ATP have the same qualitative appearance as that of ITP, which suggests that it is competing with the 6-NH_2 group of ATP for the group X in the protein chain. The presence of DNP as the phenoxide ion at *p*H 7 supports this idea. The DNP and 6-NH_2 will both, therefore, be in their basic forms in this solution, whereas the 6-OH of ITP will be present as the uncharged acid. The role of DNP is to release the constriction caused by the attraction between the 6-NH_2 and group X. Moreover, the model readily explains why DNP does not activate the myosin-catalyzed hydrolysis of ITP (Greville and Needham, '55). Since there is no attraction of the —OH group for the side chain, the DNP cannot release this inhibition, and hence there is no effect on the rate. Third, the model allows an explanation of a wide variety of apparently unrelated and confusing phenomena on a simple basis. For example, the observed activations of myosin-ATPase activity by low concentrations of *p*-chloromercuribenzoate and ethylenediaminetetraacetic acid (EDTA) also can be rationalized on the basis of a competition with the 6-NH_2 for the group X on the protein chain. Moreover, EDTA activates ATP but has a negligible effect on ITP (Bowen and Kerwin, '54) as would be predicted by this mechanism.

Although theories that explain existing anomalies are pleasant, those that suggest new experiments are pleasanter. I should like, therefore, to conclude with a description of experiments that give us some clue to the role of enzyme activators and perhaps even of hormones. Let us look for a moment at figure 4 in the light of the denaturation theory of protein "breaks" and ask which form of the protein is denatured. If we start with the enzyme at 0°C. and extrapolate along the straight line to the expected rate at 30°, we will see that this is much higher than the rate actually observed for this mechanism. Our conclusion from this comparison would be that the high-temperature form of the enzyme is clearly the dentured form. If we start our studies at 30°C., however, and extrapolate linearly from the initial rates observed there to 0°, we will find that the observed rate at zero is far less than expected. Our conclusion from this second extrapolation, therefore, would be that it is the enzyme at 0° that is the denatured form! This apparent dilemma is only semantic but it emphasizes an important conclusion; i.e., that the folding of the protein is not optimum at any temperature. A reagent that could improve this folding would therefore catalyze the enzyme activity even though it had no function in bond breaking or electron polarization. Such a role was proposed for DNP in the preceding model, and such a role may well be played by other activators in this and other systems.

A particularly intriguing activator of the myosin system is actin. This protein is not only essential for contraction but also greatly accelerates the rate of myosin-ATPase activity in the presence of Mg^{++}. Chappell and Perry ('55) originally observed a DNP-actin competition, and this was confirmed in our laboratory. It seemed logical to expect that part of the actin function, therefore, would be to loosen the 6-NH_2 protein bond in the same way as postulated for DNP. To support this possibility, Levy and Sharon measured the actin-ATP-myosin temperature curve, and the results are shown in figure 7. The similarity to the ITP and DNP-ATP

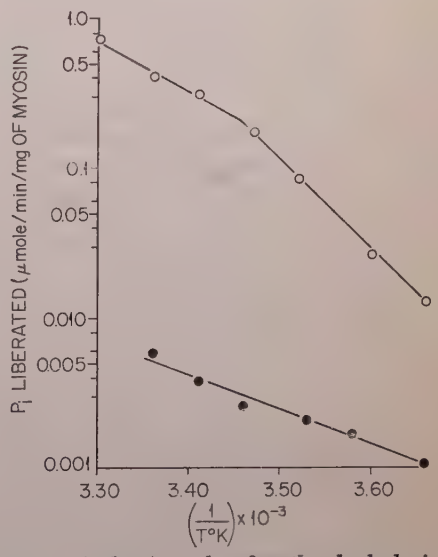

Fig. 7 Arrhenius plot for the hydrolysis of ATP by myosin (●) and actomyosin (○). Myosin conditions given in figure 1. Actomyosin conditions were: 0.025 *M* KCl, 0.005 *M* $MgCl_2$, 0.025 *M* Tris *p*H 7.3, 0.002 *M* ATP.

curves is striking. Again, the rate is accelerated when actin is added and a transition from one protein form to another is indicated, the transition seeming to occur at about 16°C. The concordance is at least a strong indication that one of the activating properties of actin is its effect on the conformation of the myosin active site (Levy *et al.*, '59b).

The previous discussion has involved two activators of the myosin system. One of these, actin, is a protein just as are a number of the already-known hormones like ACTH, and the other is a nonprotein chemical, DNP, which in many ways is analogous to thyroxine. It is tempting to speculate, therefore, that the hormones may act not as coenzymes or intermediates but as materials that favorably alter the conformation of the active sites of the appropriate enzymes.

The accumulated evidence indicates that some enzymes must have a flexible active site that plays a key role in enzyme action. Whether all enzymes must show this flexibility at the active site is a logical question. It is conceivable that they do since, as mentioned, an induced-fit type of behavior can explain the failure of both larger and smaller molecules to react. However, general studies on proteins indicate a rather wide range in flexibility. The most logical prediction at this time would seem to be that many enzymes will have a flexible active site and hence will have an induced-fit type of specificity behavior. Other enzymes will have an active site of considerable rigidity and will therefore show template-type specificity. How many enzymes will fall in each category remains to be seen, but the theory predicts a correlation between specificity pattern and protein structure that should lead to exciting new developments.

SUMMARY

Examination of the specificity behavior of a number of enzymes indicates that their action cannot be explained by the postulation of a rigid template but can be explained by the assumption that the substrate induces a change in the protein conformation at the active site and that this change is necessary for the proper alignment of catalytic groups. The temperature dependence of myosin strongly supports this conclusion, for it appears that a protein conformation change that affects the rate of hydrolysis of ITP has no apparent effect on the rate of ATP hydrolysis. A model explaining this peculiar temperature dependence can be constructed if it is assumed that the active site of the enzyme is flexible and this model, moreover, leads to an explanation for the roles of DNP and actin and their interrelations with substrates. These considerations lead to a suggestion that many enzyme activators act through their influence on the protein conformation and flexibility rather than through a direct effect on the substrate molecule.

OPEN DISCUSSION

BRUICE[2]: There would seem to be an alternate explanation to Dr. Koshland's temperature effects. There is certainly one thing that we know nothing about, and that is the process of the bond-breaking steps within the enzyme—substrate complex. Ignoring now the formation equilibria and directing our attention only to the enzyme—substrate complex, we may suppose that we have chemical equilibria occurring within the complex where bonds are being made and broken. A possible example is given in equation (8) (fig. 8). Equation (9) then gives the consequent over-all rate of the decomposition of the enzyme complex. Now if these equilibria had different heats you might expect that at some stage one would overtake the other and a plot of k_{obs} versus 1/T would bend.

$$ES_1 \overset{K_1}{\rightleftharpoons} ES_2 \overset{K_2}{\rightleftharpoons} ES_3 \xrightarrow{k\ \text{rate}} E + \text{PRODUCT} \qquad (8)$$

$$k_{obs} = k_r \left[\frac{K_1 K_2}{K_1 (K_2 + 1) + 1} \right] \qquad (9)$$

$$k_{obs} = \left[\frac{k_{rate} K_2}{K + 1} \right] \left[\frac{K_a (K_2 + 1)}{K_a (K_2 + 1) + a_H} \right] \qquad (10)$$

Figure 8

[2] T. C. Bruice, Johns Hopkins School of Medicine.

Another example that might be pertinent for the hydrolytic enzymes would be found in the instance where K_1 was actually an acid dissociation constant (K_a') [eq. (10)]. It follows then that the value of the apparent pK_a' of the participating group (determined say from a plot of k_3ES versus pH) would not be the true value but $K_a(K_2 + 1)$. This may actually be the case in chymotrypsin for, as I recall from Dr. Neurath's paper, the group participating in chymotryptic activity exhibits pK'_{app} values one unit apart in the acylation and deacylation steps.

KOSHLAND: I believe the proposed alternative will simplify to the same kinetics as the consecutive-step mechanism, but I would have to derive the formula before I was sure. [Note added in proof: On appropriate derivation, it is true that the over-all observed kinetics for the alternative proposed by Dr. Bruice are the same as the consecutive-step mechanism when ΔH is positive. However, the mechanism proposed by Dr. Bruice allows a negative ΔH, which can therefore explain curves that are concave upward. Hence the argument that was used against the consecutive-step mechanism does not hold for this alternative. We are still left, however, with the necessity to explain the difference in these "catalytic" ΔH's of ATP and ITP, and I believe that a change in protein conformation seems like the most reasonable explanation.]

TODD[3]: Leaving for the moment equations and curves, I would like to return to the more empirical part of Dr. Koshland's discussions where he was drawing a lot of wiggles on the blackboard. These encouraged me to mention some rather preliminary experiments Dr. Cramer and I did some time ago and that we have only now belatedly begun to think about following up further. What we were interested in was the idea I mentioned in the discussion of an earlier paper, that the function of an enzyme protein might be in part to localize ionization in compounds like ATP so as to vary the reactivity at different parts of a polyphosphate chain. In trying to think how we might demonstrate this, we reckoned that substrates probably do not just lie on the surface of the enzyme protein, but that the active groups might well be included in holes or depressions in the enzyme protein, at any rate in part. So we decided to look at the Schardinger dextrins, which, as you know, are capable of forming inclusion compounds. I have not got the experimental figures with me and I cannot remember them precisely, but I can tell you roughly what the results were. We found, for example, that if you take ATP and put it into solution at, say, pH 7–8 and just observe its hydrolysis you find that first one phosphate comes off, leaving ADP, which, in part, slowly breaks down to AMP. If to an ATP solution at the same pH you put in one of these dextrins, you observe first that an actual inclusion compound is formed, and second that, although there is not a very marked increase in the rate of removal of the first phosphate, there is a very much faster conversion of the ADP produced to AMP. This, of course, fits with the picture obtained from the change in absorption spectrum in adding the dextrins to ATP solutions, which indicates that it is the nucleoside part that is actually included and not the other end of the molecule.

We did somewhat similar experiments with P_1—P_2—diphenylpyrophosphate, which is a pretty stable compound in water of about pH 8 and only undergoes very slow partial hydrolysis when calcium ions are added, but if you take the same substance in solution at the same pH with or without calcium ions and add a cyclodextrin, you get an inclusion compound and complete hydrolysis to phenylphosphoric acid. These experiments are of course very crude, but we are inclined to think that there is an analogy between this and the action of an enzyme protein. The cyclodextrins are, of course, rather poor examples and suitable protein molecules might show the effect to a much greater degree, perhaps because of their much greater hydrogen-bonding propensities. This kind of inclusion effect could, of course, explain a part, at any rate, of the specificity of an enzyme, because the degree to which ionization of a polyphosphate is affected by inclusion will deter-

[3] Alexander Todd, University Chemical Laboratory, Cambridge, England.

mine to what extent the phosphate groups in a polyphosphate will be labilized and so determine with which type of compounds it could react with and with which type it would remain inert.

I thought I would mention these points because they are in some senses not dissimilar from some of the things that Dr. Koshland was saying about myosin. To put things another way, his results might mean that, under certain conditions, the stability of inclusion compounds of ITP and ATP differs. That kind of difference, I think, would account for his results without postulating any serious change in the protein.

KOSHLAND: I would agree with much of what Dr. Todd has said and am certainly very intrigued by these experiments with Schardinger dextrin. Dr. Todd's explanations support the argument that the proper orientation of catalytic groups is important in nonenzymic as well as enzymic catalysis and this, of course, pleases me very much. However, the problem that we had to explain in the case of myosin was why one of the curves gave a curvature in the region of 16°C. and the other did not; and this, I think, is difficult to explain unless a change in the structure of the protein is invoked. This change in protein structure was postulated to cause a slightly different orientation of the catalytic groups and in that sense agrees nicely with Dr. Todd's conclusions from his Schardinger dextrin work.

TODD: I do not wish to argue the point too much, since I only made a speculative observation, which would need to be thought through much more carefully.

FRENCH[4]: Since the Schardinger dextrins and their inclusion compounds have been brought up, I would like to comment on the interesting observation of their uses as model systems of enzyme action. The Schardinger dextrins, when they form inclusion compounds, do not form these compounds by hydrogen bonding with the included molecule, but rather by what the protein chemists call "hydrophobic" bonding. That is why the inclusion of diphenylpyrophosphate involves the ring rather than the phosphate part of the molecule.

A model of the cyclodextrin shows that the interior of the ring is populated by CH groups and is devoid of —OH groups. The —OH groups are primarily projecting from the sides and the periphery of the cyclic molecule. I think that this raises an interesting possibility in that the substrate of this enzyme model is held quite rigidly in proximity to the hydroxyl groups. The —OH groups could be thought of as being analogous to reactive groups of enzymes, and just by virtue of proximity they have many more opportunities for reaction with bonds that are eventually going to be broken than they would if the two molecules were not held together in this manner.

PIGMAN[5]: Dr. Koshland I wonder if you do not have two distinct situations—one in which the substrate is a small molecule and the other in which it is a large molecule. These two situations are very different. For the large molecule I can conceive of a strong effect on the protein confirmation. But for the small molecule the distances involved are only several peptide units. Isn't there enough rigidity in the protein structure, over a very limited range of two or three peptide units, to prevent this type of orientation of the active group?

One more point—in the mechanism that you proposed I noticed that you put the active site of the enzyme at the end of the substrate molecule. With many enzymes two regions seem to be involved. They attach on both sides of the bond that is being split. If it is a glycosidic bond, for example, the sugar and the aglycon are both attached. To me, this point is very fundamental. Is there any reason you put the active site at the end of the substrate molecule?

KOSHLAND: In answer to the first question, I would say that there is not enough rigidity. In fact, our argument is that a flexibility in this limited range is essential for the specificity we observe. Since the bonds that are being formed and broken are very close to each other, it is actually easier to explain specificity for a small molecule than a large one, for it is easier to see that a fit in the immediate neighborhood of the catalytic groups would have

[4] Dexter French, Iowa State College.
[5] Ward Pigman, University of Alabama Medical-Dental Schools.

more influnce on orienting these groups than in the case of more distant interactions. For large molecules we must postulate some type of sensitive spider web in which groups far from the active site can still cause appreciable change in the geometry of the catalytic amino acids. Although this is not an entirely easy concept to accept, it is certainly easier than the alternative template type of argument. In regard to your second question, the diagram is entirely schematic and would be equally true if the bond being broken had been located in the middle of the molecule instead of the end.

PIGMAN: I thought that your argument was that a substrate really sets the pattern for the enzyme molecule.

KOSHLAND: No, it sets the pattern for the active site. It does not really care what happens to the rest of the molecule. In fact, in some cases like papain, you can remove 120 amino acids without loss of enzyme activity; so it seems reasonable that 120 amino acids might coil up also without affecting the active site.

MOUNTER[6]: There are two observations that I think may be relevant to this problem of the role of the protein. In cholinesterases I was interested in the effect of substrate size. We had the acetyl, the propionyl, the chloroacetyl, and the butyryl esters. Here we have two isosteric molecules. When we studied the temperature dependence of the hydrolysis of these four substrates we got this same type of broken curve. But the striking thing was that, although the turnover numbers were very different, essentially we had the same activation energy for each case (as measured at the limting rate). I thought, therefore, that this probably was a function of the configuration of the protein rather than the reactivity of the group. The other point is the work that I mentioned before, which Mr. Angleton has just started in my laboratory, on the inactivation of cholinesterases by radiation where we find differences in inactivation with different choline esters, namely, acetylcholine, propionylcholine, and acetylmethylcholine. The interesting point to me is that the inactivation appears to be less the higher the affinity for the substrate. This very closely parallels the recent publication of Augenstine, who has shown in the case of trypsin that there is a difference in the inactivation in solution of the esterase and the protease activities, which can be correlated with breaking off two or three hydrogen bonds. It seems to me that probably we have a similar situation here, that a high affinity substrate is able to pull back more of the damaged molecule into the active configuration than that with a low specificity.

COHN[7]: I should like to ask Dr. Koshland if he has investigated the ATP hydrolysis with any of the fragments of myosin. He really has a unique opportunity here because this protein is split by proteolytic enzymes into two fragments, one of which has just as high activity as the original for ATP hydrolysis and the other has no activity. The behavior with the active fragment of myosin may supply crucial evidence on the validity of this mechanism.

KOSHLAND: Yes, we have. Dr. Yount has started some work on this and, although it is not by any means finished yet, we do have evidence of a change in specificity in the heavy meromyosin. The changed specificity of this active fragment, therefore, supports the argument that amino acids quite distant from the active site do have influence on the arrangement of amino acids in this critical region.

MAAS[8]: As a biologist I should like to make a comment, though I am not quite sure that it is entirely relevant to Dr. Koshland's discussion. As you are aware, there can be mutations for almost any enzymic reaction and in most of the cases that have been described the enzyme activity is absent. There are other kinds of mutants, so-called temperature-sensitive mutants that are somewhat different in that they have a growth requirement at a higher temperature (37°C.) but not at a lower temperature (25°). It may not be so well known that there are temperature-sensitive mutants for most reactions for which there are the "ordinary" mutants.

In a few cases the enzyme affected by mutation in temperature-sensitive mutants has been studied in detail, and it has been

[6] L. A. Mounter, Medical College of Virginia.
[7] Mildred Cohn, Washington University, St. Louis.
[8] W. K. Maas, New York University.

found that, as a result of mutation, a particularly heat-labile enzyme protein is obtained; here the mutation has resulted in the formation of an altered protein molecule. More recently it has been shown in other types of mutants that the mutation affects the quality of the enzyme produced.

Yura ('59) described an interesting case involving a temperature-sensitive mutant with a block in the last step in proline synthesis. He found that this mutant produces an altered heat-labile pyrroline 5-carboxylate reductase. Furthermore, he showed that the heat of activation of the reaction was also affected by the mutation, being three or four times as high as the heat of activation of the reaction in the wild type strain; yet the affinity of the enzyme for the substrate was not changed in the mutant. It seems to me that here the active site probably is still the same but somehow now the secondary or tertiary structure of the enzyme has been affected.

BERNHARD[9]: I have been very interested in models of how to keep little things out of big sites. Dr. Koshland showed one such mechanism today, and I should like to comment on one specifically, just because it is rather clear cut. Essentially, the structures we were dealing with are shown in figure 9, where the double-ring com-

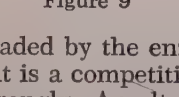

Figure 9

pound is degraded by the enzyme and the other is not (it is a competitive inhibitor), yet it binds strongly. An alternative to Dr. Koshland's explanation (and I wonder whether he has a way of distinguishing this) is to say that the site that binds the one ring can bind a ring in many ways. It is only the double specifications of the substrate that force a *specific* mode of attachment of this molecule to the enzyme surface. It is only with the two rings that there is specificity for reaction. Other molecules lacking one of the hydrophobic groups could bind to the enzyme by a large number of nearly equivalent surface interactions, and only one (a possibly unlikely situation) might lead to reaction although all the others might be strongly bound. One way of keeping little molecules out of reactions is to lead them into the site but not show them the proper route to reaction.

KOSHLAND: I think Dr. Bernhard's alternative is possible in some cases but certainly not in all. It is difficult to believe that there are so many different possible positions for the water molecule that its chance of reacting after it gets to the active site is essentially zero unless it is properly oriented. Yet as I stated in my paper, it is the failure of water to react in many cases that led us to the suggestion of an induced fit. The ATP-ITP case just discussed would also, in my opinion, be very difficult to explain on the basis of Dr. Bernhard's model.

HESTRIN[10]: The theory of "induced fit" proposed by Dr. Koshland might have implications in relation to the problem of the role of the inducer in adaptive enzyme production. It is commonly accepted nowadays that the inducer does not provide the cell with new information for a *de novo* protein synthesis, but that it stimulates a synthesis that would have occurred, though to a lesser extent, even in the absence of the inducer. However, the hypothesis presented by Dr. Koshland emphasizes that a protein could have different inducible surface configurations, and it is conceivable that once a configuration suitable for a certain substrate is induced that configuration could be made permanent by some secondary reaction. This would mean that the inducer provides information and determines the pattern of the "informed" protein. Thus we are brought back to old ideas about the analogy between antibody response and adaptive enzyme production. In our laboratory, Dr. G. Avigad has studied the influence of a group of α-glucosides acting as inducers on the nature of the α-glucosidase produced by yeast. He has found that with maltose as inducer in a suitable genotype you get an α-glucosidase whose activity toward α-methylglucoside is very small as compared to its activity toward

[9] S. A. Bernhard, National Institutes of Health.
[10] Shlomo Hestrin, The Hebrew University, Jerusalem.

maltose. However, with α-methylglucoside as inducer in the same genotype you get an α-glucosidase that does not cleave maltose though it is active toward α-methylglucoside. This is a case that I think might perhaps be interpreted most convincingly in terms suggested by Koshland's "induced-fit" type of mechanism.

It would seem from this work that maltoses in yeast can exist in a variety of forms. Presumably these are all variants of a single protein whose final form is a function both of the inducer used and the genotype involved. It is conceivable that in this system the inducer is actually shaping the surface of the protein. What is involved, however, is not an easily reversible alteration consisting of only a change in conformation but a more permanent alteration based on an induced conformational change that is made permanent as a result of a subsequent chemical reaction between peptide chains in the enzyme.

SCHREINER[11]: I should like to ask Dr. Koshland one question with respect to a heat-stable inorganic pyrophosphatase with an optimum temperature of 65° or 70°C. that shows a break in the ΔH of activation at about 45°C. Inorganic pyrophosphate is a very small substrate molecule and I wonder if you would like to comment on this observation. The work on this enzyme has been done by Militzer and Marsh at Nebraska.

KOSHLAND: I would say that it would fit into the same pattern. Along these lines, I might add that you might expect to have fewer and fewer changes in the coiling that do not affect the active site as you go to smaller and smaller enzyme molecules. With a molecule like myosin, having a molecular weight of 400,000, large areas can probably fold and unfold without affecting the catalytic activity. On the other hand, an enzyme molecule of small molecular weight would have few such changes that were not directly related to activity and, hence, these enzymes might be the best ones to attempt a correlation of enzyme activity with some structural parameter such as viscosity.

[11] H. R. Schreiner, The Linde Company, Tonawanda Laboratories.

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Summarizing Remarks

PHILIP HANDLER
Duke University School of Medicine, Durham, North Carolina

The wealth of material presented during this conference does not readily permit a brief summary. Nevertheless, a few simple points are worth noting. Perhaps most impressive is the very timeliness of this conference. In the year 8 of the First French Republic (1800) the Institute offered a gold medal and a prize equivalent to 1 kilogram of gold for the best answer to the question "What are the characteristics by which animal and vegetable substances which act as ferments can be distinguished from those which they are capable of fermenting?". This prize was never awarded and only now have we begun to define the terms in which the proper reply to this question may be couched. In general, two approaches to the problem are being assayed. For the first days of this conference we were concerned not with what an enzyme does, but rather with the task that it is called upon to perform. The reactions that proceed in biological systems under the influence of enzymic catalysts are as diverse as the interaction of organic compounds in the laboratory. Only recently has the newer knowledge of electronic mechanisms of organic chemical reactions been extended to the reactions encountered in biological systems. With increasing frequency these are described in such terms as "electrophilic," "nucleophilic," or "backsided attack." In the main, however, these formulations have, of necessity, described the reaction occurring between the participating substrates, with almost no treatment of the role of the enzyme in promoting the observed reaction. This should not be discouraging. Before we can address ourselves to the mechanism of an enzymically catalyzed process, we must have knowledge of the task confronting the specific enzyme, and it is in this sense that considerable progress has been made. The details of several such studies were presented on the first days of this conference.

Relatively little progress, however, can be recorded with respect to the role of the protein. Nevertheless, it is cheerful to note that the day seems to be at hand when such progress can be expected. Clearly, such understanding depends on our previous understanding of the structure of the catalyst itself. In only a few instances has the primary structure of an enzyme been ascertained in some detail and these are restricted to hydrolytic enzymes such as ribonuclease, trypsin, and chymotrypsin. Still, the long known sensitivity of enzymes to their environment, and the ease of thermal denaturation have always indicated that the catalytic properties of enzymes are inherent in their secondary and tertiary structure as well. But about the details of such structure almost nothing is known at the present time and, until such information is at hand, no satisfactory answer will be available to the question posed in 1800.

Ever since Emil Fischer, biochemists have harbored the concept that on each enzyme there is a site (or sites) on which the substrate must be specifically affixed and that, in some manner, binding of the substrate to that site in turn makes possible the specific reaction catalyzed by that protein. The enormous body of information with respect to enzyme specificity that has been amassed in the years since has fully substantiated this concept. Identification of such an active site on a specific enzyme is the task to which some of the last speakers have addressed themselves and it is apparent that this goal has not yet quite been achieved. Indeed, the closer it has been possible to "focus down" on such an active site the fuzzier have its dimensions become. The roles that have been suggested for histidine, serine, and aspartate residues in enzymic hydrolyses have yet to be definitively established. The efficiency of such catalysis, however, clearly

suggests that the "active site" is something more than these residues *per se* but relates to the conformation, charge distribution, polarity, and dimensions of the surrounding and adjacent structures. But this should in no wise deter those who are addressing themselves to this problem. In this regard we should note the existence of a group of enzymes, not considered at this conference, whose active sites are already labeled in nature, i.e., the oxidative enzymes that are isolated with relatively stably bound prosthetic groups, such as flavinadenine dinucleotide, heme, or a metal. Only in cytochrome c has the surrounding or adjacent peptide structure been identified. These should afford excellent opportunities for determination of the role of the protein carriers since the binding sites should be capable of definition with some certainty. Moreover, several enzymes, e.g., cytochrome c and xanthine oxidase, can be isolated pure and in quantity sufficient for such studies. It is abundantly clear that the role of the protein in such enzymes is something more than merely to alter the redox potential of its electron-accepting prosthetic group, but that role has not yet clearly been defined.

Withal, I think you will agree that the time is at hand when a concerted and vigorous attack may be made on the problems posed at this conference and that the day may well be at hand when we may begin to frame a reply to the question posed by the French Academy in 1800.

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